12 Laboratory hematology

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Introduction 316
Laboratory hematology tests 316
Hemostasis testing 330
Bibliography 346

Introduction

Diagnosis of hematologic disorders requires close clinicopathologic correlation, and laboratory testing plays an important role. Hematology laboratory tests should be ordered and interpreted within the context of a specific patient's clinical circumstances. Unexpected results can be due to preanalytical (specimen collection and processing), analytical (test performance), or postanalytical (interpretation) errors. Examples of preanalytical errors, which are common, include clotted or hemolyzed samples due to collection technique, incorrect anticoagulant (incorrect tube type), specimen mislabeling, or other issues. Common blood collection tubes used in hematology and coagulation are listed in Table 12-1.

Laboratory hematology tests

Automated blood cell counting

In addition to complete blood counts (CBCs) and the traditional 5-part leukocyte differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), newer hematology analyzers can also provide quantitative and qualitative information about reticulocytes, nucleated red blood cells (NRBCs), immature granulocytes, and platelet parameters, such as platelet immaturity. Because of the large number of cells counted and analysis using multiple physical principles and sophisticated software (Figure 12-1; most analyzers use at least 2 techniques), hematology analyzers produce accurate and precise CBCs and leukocyte differential counts, with the exception of basophils because of their low numbers. Many laboratories no longer report band neutrophils because accurate and precise identification by automated and morphologic techniques is poor, and their clinical significance (if any) appears minimal, with the possible exception of neonatal sepsis and febrile children with sickle cell disease (to identify bacterial infection in the setting of vaso-occlusive crisis). In most clinical circumstances, total white blood cell (WBC) count and automated absolute neutrophil count are better tests for identifying infection. Hematology analyzers provide excellent sensitivity to distinguish between normal and abnormal samples via operator alerts (flags) prompting microscopic review of a stained peripheral blood film for selected samples.

The online version of this chapter contains an educational multimedia component on von Willebrand disease.

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Table 12-1 Common blood collection tubes used in hematology and coagulation

Tube	Anticoagulant	Common uses	Other information
Purple (lavender) top	EDTA Anticoagulates via calcium chelation (inhibits calcium-dependent coagulation reactions) Sprayed onto the tube and does not dilute the specimen	Hematology (such as CBC with WBC differential)	Not used for coagulation testing because potent calcium chelation impacts clotting times
Light blue top	Sodium citrate Anticoagulates via calcium chelation (inhibits calcium-dependent coagulation reactions) Liquid anticoagulant (1 part citrate to 9 parts blood when tube is filled to the fill line)	Coagulation studies (such as PT, aPTT, fibrinogen, D-dimer, anti-Xa, etc)	3.2% and 3.8% sodium citrate are not interchangeable (differences in calcium chelation affect coagulation reactions), most labs require 3.2% Tube must be filled to the fill line to control calcium chelation Alternative tube for platelet counts in patients with EDTA induced pseudothrombocytopenia (results must be corrected by 10% to compare with EDTA reference interval since citrate dilutes the sample and EDTA does not)
Light yellow top	ACD (acid citrate dextrose) Anticoagulates via calcium chelation	Special studies/procedures requiring whole blood (blood bank cell storage and procedures, HLA typing)	ACD solution promotes cell survival (such as red cell units stored in ACD solution)
Red top	None (no additive or clot activator) Blood clots to create a serum sample	Chemistry (such as complete metabolic panel), serology, immunology (antibody studies)	Some red top tubes contain a clot activator, such as thrombin, to speed up the clotting process Serum cannot be used for coagulation testing (fibrinogen is consumed and other coagulation factors may be altered when blood sample is clotted to form serum)
Green top	Heparin Anticoagulates via thrombin inhibition	Stat and routine chemistry (such as complete metabolic panel)	Chemistry performed on heparin plasma samples has faster turnaround times because it eliminates the processing steps needed to clot the blood to form serum (if a red top tube is used) Heparin plasma cannot be used for coagulation testing because thrombin inhibition impacts clotting times

Aperture impedance (Coulter principle)

In this classic method, cells diluted in a conducting solution are counted, and their volume is determined by measuring change in electrical resistance as they flow through a narrow aperture and interrupt a direct electrical current. Software analysis defines red blood cells (RBCs), WBCs, and platelets based on volume limits and calculates RBC and platelet indices.

Optical absorbance

This technique exploits the cytochemical reaction of an intracellular enzyme, such as myeloperoxidase, to absorb white light from a tungsten light source after the addition of a substrate. Light absorbance is proportionate to the intensity of the enzyme-catalyzed reaction. This technique may be used to detect and distinguish peroxidase-containing cell types (neutrophils, eosinophils, monocytes) from peroxidase-negative lymphocytes and basophils.

Optical light scatter

This method monitors the light-scattering properties of blood cells using a technique similar to that employed by flow cytometers. Cells pass in single file across the path of a unifocal laser. The amount of light scattered at a low angle from the incident light path is proportional to cell volume. The amount of light scattered at a wide angle depends on such factors as cytoplasmic granules and nuclear complexity. All of the major hematology analyzers use light-scattering technology.

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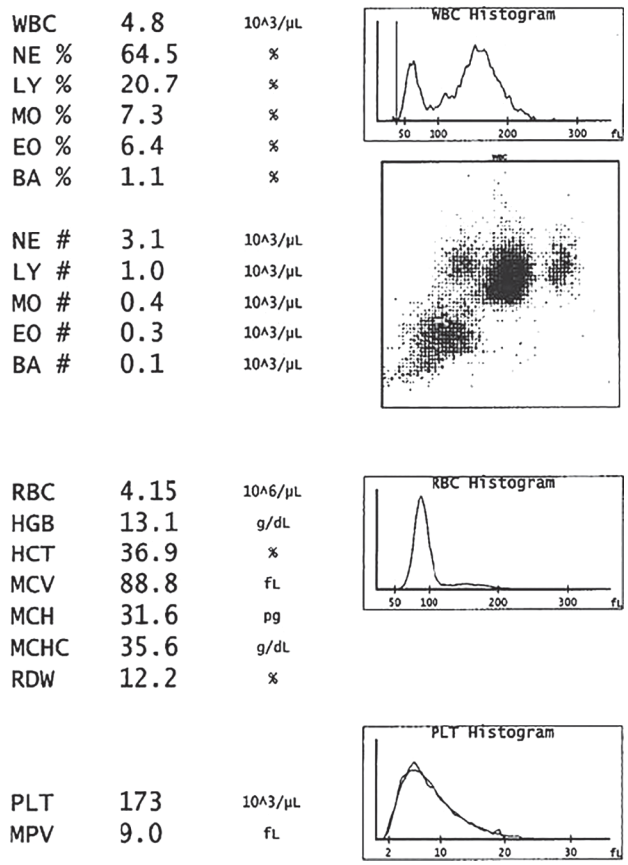

Figure 12-1 Data and histograms performed on a Beckman-Coulter automated hematology analyzer from a healthy adult. The WBC, RBC, and platelet (PLT) histograms represent cell volumes determined by impedance. The second histogram from the top displays WBC light scatter in a flow cell; the y-axis indicates forward scatter and volume, and the x-axis indicates side scatter due to granularity and nuclear features. Basophils (BA) are detected by an alternative physical property not displayed. EO, eosinophil; HCT, hematocrit; LY, lymphocyte; MO, monocyte; NE, neutrophil.

Fluorescence

In addition to the physical properties of cells, fluorochrome-labeled antibodies recognizing cell surface or intracellular epitopes (after cell permeabilization) and fluorescent dyes further refine the separation of individual cell types. A variety of reagents can be used to distinguish platelets (thiazole orange, anti-CD41, anti-CD42b, and anti-CD61), reticulocytes (thiazole orange, anti-CD4K, and RNA dyes), fetal RBCs (anti–hemoglobin F [fetal hemoglobin]), NRBCs (propidium iodide, Draq 5, and other DNA-binding dyes), neutrophils, lymphocytes, and blasts.

Erythrocyte analysis

Automated blood cell counters measure the number (RBC count, reported in units of 10^{12}/L [10^6/µL]) and volume (mean corpuscular volume [MCV], reported in units of fL, or 10^{-15} L) of RBCs and hemoglobin concentration (reported in units of g/dL after lysing RBCs); all other parameters are typically calculated from these measurements, but this is instrument dependent. Hemoglobin is converted by potassium ferricyanide to cyanmethemoglobin, and absorbance is measured by a spectrophotometer at 540 nm. Some analyzers use a cyanide-free method. RBCs may be spuriously increased in patients with marked hyperleukocytosis (since WBCs are included in the RBC count and their numbers normally differ by a factor of >500) and giant platelets (sized similarly to RBCs) and spuriously decreased in the presence of RBC agglutinins (multiple cells counted as one) and in vitro hemolysis. Hemoglobin measurements can be elevated artifactually by increased sample turbidity because of leukocytosis, paraproteinemia, carboxyhemoglobinemia, hyperbilirubinemia, or hyperlipidemia. Sulfhemoglobin also interferes with hemoglobin values.

MCV is determined from the distribution of individual RBC volumes. This measurement can be elevated artificially by agglutination of RBCs, resulting in measurement of more than 1 cell at a time; hyperglycemia, causing osmotic swelling of the RBC; and leukocytosis (due to inclusion of WBCs). In general, interferences in measured RBC parameters are analyzer dependent and also affect calculated values that use the affected information.

Automated hematocrit (%) is typically calculated by multiplying the MCV by the RBC number: hematocrit = MCV (10^{-15} L) × RBC count (10^{12}/L) divided by 10 (proportion [%] of the blood volume occupied by red cells). Some analyzers directly measure hematocrit and use it to calculate other values.

The mean corpuscular hemoglobin (MCH) is expressed in picograms (10^{-12} g). The MCH is calculated by dividing hemoglobin (g/L) by RBC count (10^{12}/L). An elevated MCH can be an artifact of increased plasma turbidity due to artificially increased hemoglobin. If RBC hemoglobinization is normal, MCH reflects cell size (macrocytic cells have a higher MCH than normocytic cells, which may generate a high flag).

The MCH concentration (MCHC) is expressed in grams of hemoglobin per deciliter of packed RBCs. The MCHC is calculated by dividing the hemoglobin concentration (g/dL) by the hematocrit (%) × 100. Because it reflects a concentration, MCHC captures whether hemoglobinization is appropriate for cell size. Any artifact affecting the hematocrit or hemoglobin determinations (as described previously) can alter the MCHC.

The RBC distribution width (RDW) is the coefficient of variation of RBC volume (anisocytosis, reflects the range of RBC sizes in the sample): standard deviation/MCV × 100.

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The RDW is used in the evaluation of anemia. The RDW is more frequently increased in microcytic anemias due to iron deficiency anemia than in thalassemia or anemia of chronic disease; it is also elevated more frequently with macrocytic anemias due to vitamin B_{12} or folate deficiency compared with liver disease, hypothyroidism, or reticulocytosis. Myelodysplastic syndromes with ring sideroblasts or RBC transfusions in patients with severe microcytic or macrocytic anemias can produce a dimorphic RBC pattern with a very wide RDW.

Reticulocyte counts

Automated hematology analyzers use dyes or fluorescent techniques to detect residual mRNA in young erythrocytes, and all provide accurate reticulocyte counts expressed as a percentage of RBCs and/or as an absolute number. Some blood cell counters provide reticulocyte indices that are analogous to the standard RBC indices, including reticulocyte hemoglobin content (CHr) on Advia analyzers (Siemens, Tarrytown, NY) and reticulocyte MCV (MCVr) on several other analyzers. Reductions in CHr and MCVr reflect inadequate hemoglobin synthesis in real time, providing immediate information about functional iron deficiency when other biochemical markers of iron availability may be difficult to interpret due to inflammatory conditions. CHr is particularly useful for assessing response to erythropoiesis-stimulating agents and iron therapy in renal dialysis patients. The immature reticulocyte fraction is another parameter that measures immature reticulocytes and serves as a marker of erythropoiesis in the bone marrow, wherein very low values reflect bone marrow aplasia and high values reflect increased erythropoiesis in the bone marrow, although clinical utility is still being assessed. Corrected reticulocyte counts involve a manual calculation that compares the degree of reticulocytosis and magnitude of anemia to assess whether the bone marrow response is adequate.

Red blood cell fragments

The reliable identification of RBC fragments (schistocytes) is important in the diagnosis of microangiopathies, such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), transplant-associated thrombotic microangiopathy, and disseminated intravascular coagulation (DIC). The number of schistocytes is often greater in TTP than in DIC. The Sysmex XE and Siemens Advia systems both take advantage of the small size of RBC fragments to distinguish them from normal RBCs. Although automated platforms may slightly overestimate or underestimate the number of fragmented cells compared with manual methods, this parameter can be used by the laboratory via instrument flags for identification of specimens needing manual microscopic review. A percentage of schistocytes above 1% of RBCs is considered an indicator of a thrombotic microangiopathy. A recent International Council for Standardization in Haematology (ICSH) guideline addresses identification, diagnostic value, and quantitation of schistocytes.

Nucleated red blood cells

Circulating NRBCs occur in newborns; however, beyond this period, the presence of NRBCs is abnormal and associated with various hematopoietic stresses or marrow structural compromise, including hemolytic anemias, myeloproliferative neoplasms, metastatic cancer to the bone marrow, and hypoxia. All major hematology analyzers enumerate NRBCs and correct WBC and lymphocyte counts for interference from NRBC analysis.

Leukocyte differential analysis

To differentiate lymphocytes, monocytes, neutrophils, eosinophils, and basophils, most instruments use impedance and light scattering plus additional physical properties. Beckman Coulter and Sysmex analyzers utilize radiofrequency conductivity, and Advia analyzers use peroxidase staining. Leukocyte differentials typically are reported as percentages of WBCs and as absolute counts (percentage of cell type × total WBC count). Automated blood cell counters provide sensitive flags and warnings for immature granulocytes, including blasts, monocytes, and abnormal lymphocytes. Instrument manufacturers continue to refine technologies to more accurately quantify immature or abnormal cells that may be present in small numbers (such as blasts).

Platelet analysis

Automated blood cell counters enumerate platelets, measure volume, and calculate mean platelet volume (MPV). Counts are less accurate in patients with thrombocytopenia, especially when severe. MPV has been used to help discriminate between underproduction of platelets and increased platelet destruction, which results in bone marrow release of younger and larger platelets, but this should always be correlated with additional clinical information. Platelets undergo time-dependent shape changes when exposed to ethylenediaminetetraacetic acid (EDTA), which may lead to inaccurate MPV results and thus diminished clinical utility in laboratories where blood samples are not tested right away. Inaccurate automated platelet counts can result from fragmented RBCs that are counted as platelets (spuriously high platelet count) and congenital or acquired disorders with large

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platelets that are counted as RBCs, including EDTA-mediated platelet clumping (spuriously low platelet count due to large or clumped platelets being counted as RBCs). Hematology analyzers provide sensitive warnings for abnormal platelet populations requiring manual smear review to confirm or revise platelet counts. If spurious thrombocytopenia due to EDTA platelet clumping is present, accurate platelet counts may be obtained by recollecting the specimen in sodium citrate (although this needs to be validated by the laboratory). Analogous to reticulocytes, young platelets (also called reticulated platelets) contain detectable mRNA. Currently, only certain analyzers provide an immature platelet fraction based on the analysis of cell volume and fluorescence intensity of mRNA binding dye. Similar to use of the MPV, potential applications include differentiating thrombocytopenia due to marrow failure from peripheral destruction and determining earlier evidence of marrow regeneration following stem cell transplantation or response to a thrombopoietin-mimetic drug.

Examination of peripheral blood film

Blood smears are air-dried and typically stained with either Wright or May-Grünwald-Giemsa stains and can be prepared manually or by automated slide maker/stainers, which can be interfaced with hematology analyzers. Some analyzers (eg, the Roche Cobas m511) "print" blood onto a glass slide and stain with proprietary dyes and then derive all CBC measurements from the slide as well as generate cell images onto a computer screen. Microscopic examination or image analysis of stained blood smears can identify morphologic abnormalities that automated hematology analyzers nonspecifically flag or, rarely, miss. Microscopic examination begins at low power (×10), scanning for platelet clumps or abnormal, large, nucleated cells, such as blasts, that may be located along the lateral and leading edges of the smear. At higher magnification (×50 and ×100), the optimal area to examine RBC, platelet, and leukocyte morphologies and to perform WBC differentials is the transitional area between the thick part of the smear and the leading edge (Table 12-2) where there are only a few overlapping RBCs and central pallor of normal RBCs is evident. Hematologists should review a patient's peripheral smear as part of any consultation involving qualitative or quantitative blood cell abnormalities.

The accuracy of manual WBC differentials suffers from small sample size (typically 100-200 cells), distributional bias of WBCs on the smear, and variable interobserver agreement regarding cell classification. Advances in digital microscopy and image analysis may improve the accuracy of WBC classification while reducing technical time. For example, the CellaVision DM96 (CellaVision, Lund, Sweden) scans a stained blood smear, makes digital images of WBCs, classifies them, and presents the sorted WBC images to an operator to confirm or reclassify. When compared with manual differentials, automated morphologic differentials demonstrate excellent routine differential accuracy and sensitivity to detect blasts. In addition, stored images can be reviewed at remote locations, such as outpatient clinics, and saved for educational purposes. However, it should be noted that image analysis is performed at higher magnification, focusing on individual cells of interest, and may have limited ability to identify certain abnormalities. Laboratories using this technology should have detailed policies for when manual microscopic review of a slide is required.

Supravital stains are used to detect RBC inclusions; these are manual methods and labor intensive. Crystal violet detects denatured hemoglobin inclusions (Heinz bodies) because of enzymopathies, such as glucose-6-phosphate dehydrogenase (G6PD) deficiency; brilliant cresyl blue is used to precipitate and detect unstable hemoglobins (hemoglobin H in α-thalassemias).

The bone marrow aspirate and biopsy

The most frequent indications for bone marrow biopsy include: unexplained cytopenias; unexplained leukocytosis, including suspected acute leukemia, erythrocytosis, or thrombocytosis; staging of lymphoma and some solid tumors (particularly in patients with cytopenias or other findings suggestive of bone marrow involvement); diagnosis of plasma cell neoplasms (myeloma and monoclonal gammopathy of uncertain significance); evaluation of systemic iron levels; evaluation of an infectious process; and unexplained splenomegaly. Bone marrow aspirate and biopsy are most commonly performed by collecting specimens from the posterior iliac crest. In newborns and young infants, marrow aspirates often are obtained from the anterior tibia. Quality smears require adequate spicule harvesting because perispicular areas are the most representative areas to examine.

The bone marrow aspirate and touch preparations from trephine samples are air-dried and usually stained with either Wright or May-Grünwald-Giemsa stain. The aspirate smear is used for cytologic examination of the bone marrow cells and for performing the differential. Bone marrow core biopsies are most commonly fixed in formalin, and the biopsy specimen is decalcified and embedded in paraffin; 3- to 4-μm sections are then cut and stained with hematoxylin and eosin or Giemsa stain. Bone marrow aspirates can also be sent

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Table 12-2 Red blood cell abnormalities

Abnormality	Description	Cause	Disease association
Acanthocytes (spur cells)	Irregularly spiculated	Altered membrane lipids	Liver disease, abetalipoproteinemia, postsplenectomy
Basophilic stippling	Coarse punctate basophilic inclusions	Precipitated ribosomes	Lead toxicity, thalassemias, pyrimidine-5′-nucleotidase deficiency
Bite cells (degmacytes)	Smooth semicircle taken from 1 edge	Heinz body pitting by spleen	G6PD deficiency, drug-induced oxidant hemolysis
Burr cells (echinocytes)	Short, evenly spaced spicules, central pallor present	May be related to abnormal membrane lipids	Usually artifactual, also uremia
Cabot ring	Circular, blue, threadlike inclusion with dots	Nuclear remnant	Postsplenectomy, hemolytic anemia, megaloblastic anemia
Howell-Jolly bodies	Single, small, discrete basophilic dense inclusion	Nuclear DNA remnant	Postsplenectomy, hemolytic anemia (acute), megaloblastic anemia
Ovalocyte (elliptocyte)	Elliptically shaped cell	Abnormal cytoskeletal proteins	Hereditary elliptocytosis
Pappenheimer bodies	Small dense basophilic granules of varying size; multiple	Iron-containing siderosomes or mitochondrial remnant	Sideroblastic anemia, iron overload
Schistocytes (helmet cells)	Distorted, fragmented cell, with 2-3 pointed ends, loss of central pallor	Mechanical distortion in the microvasculature by fibrin strands or platelet thrombin, or intracardiac or intravascular devices	Microangiopathic hemolytic anemia due to disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, or hemolytic uremic syndrome, prosthetic heart valves, severe burns
Sickle cell (drepanocyte)	Sickle-shaped with pointed ends	Molecular aggregation of hemoglobin S	Sickle cell disorders, not seen in hemoglobin S trait
Spherocytes	Spherical cell with dense appearance and absent central pallor, usually decreased diameter	Decreased membrane surface area	Hereditary spherocytosis, autoimmune hemolytic anemia
Stomatocytes	Mouth- or cup-like deformity	Membrane defect with abnormal cation permeability	Hereditary stomatocytosis, artifact in air-dried smears (must be confirmed on a wet preparation of fresh blood)
Target cell (codocyte)	Target-like appearance, often hypochromic	Increased redundancy of cell membrane	Liver disease, postsplenectomy, thalassemia, hemoglobin C disease
Teardrop cell (dacrocyte)	Distorted, drop-shaped cell	Infiltration of bone marrow by fibrosis, granulomas, hematopoietic or metastatic neoplasms	Myelofibrosis, myelophthisic anemia

Adapted from Greer JP et al, eds, Wintrobe's Clinical Hematology, 14th ed. (Wolters Kluwer; 2019).

for microbiologic culture to work up suspected infections and for ancillary studies, such as flow cytometry, karyotype, fluorescence in situ hybridization (FISH), or molecular studies.

Immunohistochemical stains

A large array of specific antibodies detected by enzymatic formation of a colored product linked to the antigen-antibody complex are now available for use on bone marrow biopsies or other tissues. Many historic cytochemical stains, such as tartrate-resistant acid phosphatase (TRAP) and myeloperoxidase, have been converted into immunohistochemical reactions. Table 12-3 summarizes common stains used for evaluation of hematopoietic disorders in blood, bone marrow, and tissue specimens.

Immunohistochemistry (IHC) is used on marrow biopsies and clot sections as an alternative or adjunct to flow cytometry. The advantage of IHC is the ability to correlate morphology with phenotype. IHC can be used to phenotype undifferentiated tumors, lymphoproliferative disorders, and atypical lymphoid infiltrates and can assist with blast count enumeration in certain cases. In patients whose marrow cannot be aspirated (dry tap), IHC can be performed on the biopsy section. IHC also can be used on sections of lymph nodes or other tissues when there is concern about lymphoma or another neoplastic disease.

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Table 12-3 Common stains used in blood, bone marrow, and tissue specimens for evaluation of hematologic disorders

Stain	Description
Myeloperoxidase	Primary granules of neutrophils and secondary granules of eosinophils. Monocytic lysosomal granules stain faintly. Mature lymphocytes do not stain. Performed by cytochemistry, immunohistochemistry, or flow cytometry. Sudan black B (cytochemical stain) demonstrates a similar pattern.
Naphthol AS-D chloroacetate esterase (specific esterase, Leder stain)	Neutrophils and mast cells stain; lymphocytes and monocytes do not stain.
α-Naphthyl butyrate or α-Naphthyl acetate (nonspecific esterase)	Stains monocytes, macrophages, and histiocytes. Does not stain neutrophils or eosinophils. Dot-like pattern in mature T lymphocytes. Megakaryocytes stain with α-naphthyl acetate but not α-naphthyl butyrate.
TdT	Intranuclear enzyme. Stains thymocytes and lymphoblasts but not mature lymphocytes. Some myeloblasts stain positively. Performed by cytochemistry, immunohistochemistry (for fixed tissues), or flow cytometry.
TRAP	Stains an acid phosphatase isoenzyme. Positive staining in hairy cell leukemia, Gaucher cells, activated T lymphocytes. Performed by cytochemistry or immunohistochemistry (for fixed tissues).
Periodic acid–Schiff (PAS)	Detects intracellular glycogen and neutral mucosubstances. Positive staining in acute lymphoblastic leukemia, acute myeloid leukemia, erythroleukemia, and Gaucher cells.
Toluidine blue	Detects acid mucopolysaccharides. Positive in mast cells and basophils. More specific stains for mast cells are available by immunohistochemistry.
Iron	Perls or Prussian blue reactions are used to identify hemosiderin in NRBCs (sideroblastic iron) and histiocytes (reticuloendothelial iron). Ring sideroblasts are abnormal NRBCs with at least 5 blue-staining iron granules surrounding at least one-third of the nucleus. These iron granules are present in mitochondria surrounding the nuclear membrane. Performed on unfixed smears or fixed tissue sections. Iron staining of a bone marrow core biopsy can underestimate the marrow iron stores because of the loss of iron during decalcification.
Grocott's methenamine silver (GMS)	Stain for identification of fungal organisms, carbohydrates in the cell walls of fungal organisms stain brown to black.
Acid fast stains (Ziehl-Neelsen or Kinyoun methods)	Acid fast organisms have cell walls containing large amounts of mycolic acid, fatty acids, waxes, and complex lipids. Acid fast staining methods use carbolfuchsin, which is a lipid-soluble stain that penetrates the cell wall. Acid fast organisms, such as mycobacteria, are highlighted pink/red.

Preparation of bone marrow samples for ancillary studies

Bone marrow collected in EDTA is adequate for both flow cytometry and molecular analysis. Bone marrow collected for cytogenetic studies should be collected in heparin.

Paraffin-embedded tissue can be used for polymerase chain reaction (PCR) of genomic DNA sequences depending on the laboratory. Reverse-transcriptase PCR assays require that RNA preparations be performed as early as possible to prevent digestion by ubiquitous nucleases. See Table 12-4 for specimen allocation for ancillary studies based on clinical issue.

Flow cytometry

The most common applications of flow cytometry in hematology include the detection of cell surface or cytoplasmic proteins using fluorescent-labeled monoclonal antibodies and the assessment of DNA content using DNA-binding dyes.

Flow cytometry is used for phenotyping populations of cells, enumerating early progenitors for stem cell transplantation, detecting minimal residual disease, detecting targets for immunotherapy, and assessing the presence of prognostic markers.

Gating is necessary to identify cells of interest in a mixed population of cells. Three major leukocyte populations (lymphocytes, monocytes, and neutrophils) can be defined using light scatter. Forward-angle scatter (FS; low angle) measures cell size, and side-light scatter (SS) measures internal cellular granularity. Lymphocytes have the lowest FS and SS signals, monocytes have intermediate FS and SS signals, and neutrophils have high SS and slightly lower FS signals.

The most common method for gating different cell populations is by plotting right-angle SS against CD45

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Table 12-4 Specimen allocation for ancillary studies

Clinical problem	Ancillary techniques
Pancytopenia	Flow cytometry (LGL, hairy cell leukemia, PNH clone, MDS, AML) Cytogenetics (AML, MDS) Molecular genetics
Acute myeloid leukemia	Flow cytometry (blast phenotyping, monitoring/minimal residual disease) Cytogenetics and FISH (classification, monitoring) Molecular genetics, including NGS (classification, monitoring) Immunohistochemistry (assist with blast enumeration/distribution in difficult cases)
Lymphoproliferative disorder	Flow cytometry (phenotyping, prognostic markers, monitoring/minimal residual disease in B-ALL) Cytogenetics and FISH: (classification, prognostic markers) t(1;19) in B-ALL, t(14;18) in follicular lymphomas, etc (MYC, BCL2, BCL6 FISH in DLBCL) Molecular genetics (demonstration of clonality, etc) Immunohistochemistry (phenotyping, prognostic markers, immunoarchitecture)
Myeloproliferative neoplasms	Cytogenetics and FISH (diagnosis, monitoring) FISH (BCR-ABL1, PDGFR-alpha, PDGFR-beta, FGFR1, CBFB) Molecular genetics (diagnosis, prognostic markers, monitoring) (BCR-ABL1, JAK2, CALR, MPL)
Plasma cell disorders	Flow cytometry (phenotyping, labeling index, monitoring/minimal residual disease) Cytogenetics and FISH (prognosis, monitoring)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; DLBCL, diffuse large B-cell lymphoma; LGL, large granular lymphocyte leukemia; MDS, myelodysplastic syndrome; MPL, myeloproliferative leukemia.

(Figure 12-2). Cells can be separated based on the intensity of staining they display with the conjugated antibody that is classified as either bright or dim. Lymphocytes are bright CD45 and have a low SS signal, neutrophils are dim to moderately bright CD45 and have a high SS signal, and monocytes are bright CD45 and have an intermediate SS. Blasts have low SS and dim-to-negative CD45 expression, the latter being more common in blasts of lymphoid lineage.

Flow cytometry also can be used to detect other cell populations, such as natural killer (NK) cells, eosinophils, and mast cells. For instance, NK cells express CD2, CD7, CD16, and CD56 and show variable expression of CD57 and CD8. NK cells do not express CD3, and the absence of CD3 expression can be used to differentiate NK cells from T cells.

In addition to determining cell lineage, flow cytometry can be used to detect prognostic markers. For example, flow cytometric analysis of the tyrosine kinase ZAP-70 can be used to subdivide chronic lymphocytic leukemia (CLL) into prognostic groups. Positivity for ZAP-70 is highly correlated with unmutated immunoglobulin heavy chain variable region (IgV_h), a feature of CLL arising from pre–germinal center cells, and decreased overall survival when compared with patients with CLL arising from post–germinal center cells. Positivity for CD38 by flow cytometric analysis also is correlated with unmutated IgV_h, but the correlation is not as strong as it is with ZAP-70.

Uncommitted hematopoietic progenitors are CD34+ and $CD38^-$; expression of CD38 is evidence of lineage commitment. Myeloid maturation is characterized by the following maturational sequence: colony-forming units—erythroid granulocyte, macrophage, and megakaryocyte (CFU-GEMM, $CD34^+$, major histocompatibility complex [MHC] class II positive, $CD33^{-/+}$)—and followed by colony-forming units—granulocyte and macrophage (CFU-GM, CD34+, MHC class II positive, $CD33^+$, $CD13^{-/+}$, $CD15^{-/+}$). Neutrophil precursors then progressively lose MHC class II and CD33 and gain CD11b, CD16, and CD32. Monocytes retain expression of MHC class II and CD33 and also gain expression of CD14 and CD64.

Appearance of CD71, loss of CD34 and CD33, and decreased expression of CD45 characterize erythroid maturation. With further differentiation, CD71 expression decreases, glycophorin expression increases, and CD45 disappears.

Megakaryocytic differentiation is indicated by the expression of glycoprotein (GP) IIb (CD41). GPIIb/IIIa (CD61) expression increases as CD34 expression decreases. GPIb (CD42b) is expressed at the promegakaryocyte stage. GPV (CD42d) expression increases with megakaryocyte differentiation. Differential expression of CD41, CD42b, and CD61 can also be used to study platelets and diagnose platelet disorders, including Glanzmann thrombasthenia and Bernard-Soulier syndrome.

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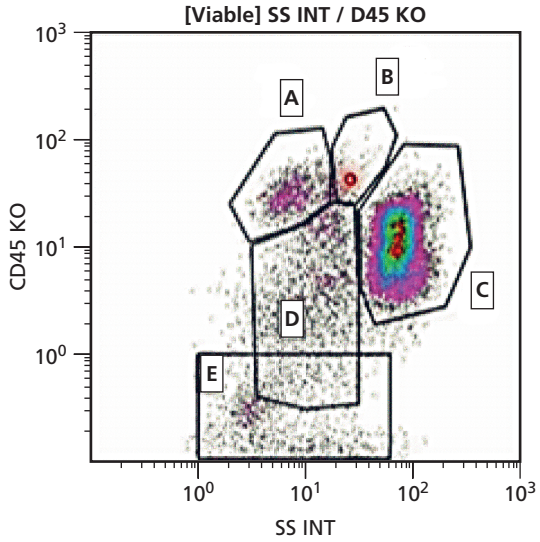

Figure 12-2 Example of peripheral blood flow cytometry gating with right angle (side scatter) displayed on the x-axis and CD45 on the y-axis. (A) Lymphocytes are bright CD45 and have a low SS signal, (B) monocytes are bright CD45 and have an intermediate SS, (C) neutrophils are dim to moderately bright CD45 and have a high SS signal, (D) blasts (few in this example) have low SS and dim-to-negative CD45 expression, and (E) debris does not express CD45.

B- and T-cell precursors express terminal deoxynucleotidyl transferase (TdT), human leukocyte antigen (HLA)-DR, and CD34. B-cell differentiation is indicated by the expression of CD19 followed by CD10. As B cells mature, they lose cell surface expression of CD34 and CD10 and express IgM on the cell surface. Expression of surface IgM is associated with the expression of mature B-lymphocyte markers (CD20, CD21, CD22, and CD79b). Mature B cells express an immunoglobulin heavy chain, such as IgM, and either the κ- or a λ-light chain. A predominant expression of one type of light chain on the cell surface of a population of B cells is known as light-chain restriction and is indicative of a monoclonal process.

T-cell precursors express TdT, HLA-DR, and CD34. Differentiation is indicated by the expression of cytoplasmic CD3 and CD7 followed by the expression of CD2 and CD5. The common thymocyte also expresses CD1a, CD4, and CD8. The mature helper or inducer lymphocyte expresses CD2, CD3, CD4, and CD5 and may express CD7. The mature suppressor or cytotoxic T lymphocyte expresses CD2, CD3, CD5, and CD8 and may express CD7. T-cell neoplasms may be associated with abnormal expression patterns of T-cell antigens, and the abnormal pattern may be detected by flow cytometric analysis. See Tables 12-5 through 12-11 for useful CD markers that can be assessed by flow cytometry (or IHC staining), depending on the lab and antibodies available for the methodology.

Flow cytometry can be used to diagnose paroxysmal nocturnal hemoglobinuria (PNH). PNH is associated with the absence of glycosylphosphatidylinositol (GPI)-anchored membrane proteins, including 2 complement regulatory molecules: decay accelerating factor (DAF, CD55) and protectin (MIRL, CD59). The absence of these proteins from the cell surface of erythrocytes can be detected by flow cytometry using antibodies to CD55 and CD59. Alternatively, PNH granulocytes are detected by the absence of GPI anchor binding by FLAER, an Alexa 488–labeled variant of aerolysin. Flow cytometry technology can discriminate between fetal and adult RBCs or Rh+ and Rh$^-$ RBCs during pregnancy and postpartum and can identify RBC cytoskeletal disorders, such as hereditary spherocytosis (HS), using eosin-5-maleimide (EMA) binding.

Cytogenetics

Cytogenetic analysis can be performed from cultured (indirect) and uncultured (direct) preparations. In the indirect assay, cells are grown so that mitotic forms can be visualized and distinct chromosomal banding patterns can be assessed (conventional cytogenetics). Growing or culturing the cells increases the mitotic rate and improves chromosome morphology. Mitogens may be useful in improving the yield of karyotyping abnormal cells and are particularly useful when analyzing mature B- or T-cell processes. A cytogenetic clone is defined by a minimum of 2 mitotic cells with the same abnormality. Constitutional chromosome abnormalities associated with either congenital genetic syndromes or normal variants are determined on peripheral blood T lymphocytes grown in culture with phytohemagglutinin, a T-cell mitogen.

Fluorescence in situ hybridization is a cytogenetic technique that uses specific fluorescent-labeled DNA probes to identify each chromosomal segment. FISH can be performed using either cultured or uncultured preparations. In the uncultured technique, the assay is performed using nuclear DNA from interphase cells that are affixed to a microscope slide. FISH can be performed using air-dried bone marrow or peripheral blood film or fixed and sectioned tissues, depending on the laboratory; decalcification typically interferes with FISH assays.

Hybridization of centromere-specific probes is used to detect monosomy, trisomy, and other aneuploidies. Chromosome-specific libraries, which paint the chromosomes different colors (spectral karyotyping), are useful in identifying marker chromosomes or structural

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Table 12-5 Clinically useful CD markers and other markers

Marker	Lineage association
Progenitor cells	
CD34	Progenitor cells, endothelium
CD38	Myeloid progenitors, T cells, B cells, NK cells, plasma cells, monocytes, CLL subset
B-cell markers	
CD10	Pre–B lymphocytes, germinal center cells, neutrophils
CD19	B cells (not plasma cells or follicular dendritic cells)
CD20	B cells (not plasma cells)
CD21	Mature B cells, follicular dendritic cells, subset of thymocytes
CD22	Mature B cells, mantle zone cells (not germinal center cells)
CD23	B cells, CLL
CD79b	B cells (not typical CLL)
CD103	Intraepithelial lymphocytes, hairy cell leukemia, T cells in enteropathic T-cell lymphoma
FMC7	B cells (not typical CLL), hairy cell leukemia
T-cell markers	
CD1a	Cortical thymocyte and certain T-cell leukemias, also positive in epidermal Langerhans cells and dendritic cells
CD2	Pro- and pre-T cells, T cells, thymocytes, NK cells, some lymphocytes in CLL and B-ALL
CD3	Thymocytes, mature T cells, cytoplasm of immature T cells
CD5	Thymocytes, T cells, B cells in CLL, B cells in mantle cell lymphoma
CD4	Helper T cells, monocytes, dendritic cells, activated eosinophils, thymocytes
CD7	Pro- and pre-T cells, T cells, thymocytes, NK cells, some myeloblasts
CD8	Suppressor T cells, NK cells, thymocytes
CD25	Activated T and B cells, adult T-cell leukemia/lymphoma
NK/cytotoxic T-cell markers	
CD16	NK cells, monocytes, macrophages, neutrophils
CD56	NK cells, myeloma cells
CD57	NK cells, T-cell subset
Myeloid and monocytic markers	
CD13	Monocytes, neutrophils, eosinophils, and basophils
CD14	Monocytes, macrophages, subset of granulocytes
CD33	Myeloid lineage cells and monocytes
CD117	Immature myeloid cells, AML, mast cells
Monocytes	
CD11c	Monocytes, macrophages, granulocytes, activated B and T cells, NK cells, hairy cell leukemia
CD15	Myeloid lineage cells and monocytes
CD64	Monocytes, immature myeloid cells, activated neutrophils
Megakaryocytic markers	
CD41	Platelets and megakaryocytes (GPIIb)
CD42	Platelets and megakaryocytes (CD42a: GPI; CD42b: GPIb)
CD61	Platelets, megakaryocytes, endothelial cells (GPIIb/IIIa)
Erythroid markers	
CD71	Transferrin receptor is upregulated upon cell activation
CD235a	Glycophorin A
Hemoglobin	Stains hemoglobin in mature red cells and precursors
E-cadherin	Stains immature erythroid precursors

AML, acute myeloid leukemia; B-ALL, B-lineage acute lymphoblastic leukemia.

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Table 12-6 Acute myeloid leukemia phenotyping using the FAB classification based on morphology

	HLA-DR	CD34	CD33	CD13	CD11c	CD14	CD41	CD235a
M0	+	+	+	+/−	+/−	−	−	−
M1	+	+	+	+	+/−	+/−	−	−
M2	+/−	+/−	+	+	+/−	+/−	−	−
M3	−	−	+	+	+/−	−	−	−
M4	+	+/−	+	+	+	+	−	−
M5	+	−	+	+	+	+	−	−
M6	+/−	−	−	−	+/−	−	−	+
M7	+/−	+/−	+/−	−	−	−	+	+

The World Health Organization (WHO) classification is now more commonly used and incorporates cytogenetic and molecular abnormalities.

FAB, French-American-British.

Table 12-7 B-lineage acute lymphoblastic leukemia phenotyping

	TdT	CD19	CD10	CD20	Cyto-μ	Surface Ig
Pro-B	+	+	−	−	−	−
Pre-pre-B (common ALL)	+	+	+	−	−	−
Pre-B	+	+	+	+/−	+	−
Early B (Burkitt)	−	+	+	+	−	+

Cyto-μ, cytoplasmic mu; Ig, immunoglobulin.

Table 12-8 T-lineage acute lymphoblastic leukemia phenotyping

Surface	TdT	CD7	CD2	CD5	CD1a	sCD3	cCD3	CD4/CD8
Prothymocyte	+	+	+	−	−	−	+	−/−
Immature thymocyte	+	+	+	+	−	−	+	−/−
Common thymocyte	+	+	+	+	+	+/−	+	+/+
Mature thymocyte	−	+	+	+	-	+	+	CD4 or CD8+
Mature T cell	−	+	+	+	-	+	+	CD4 or CD8+

cCD3, cytoplasmic CD3; sCD3, surface CD3.

rearrangements, such as translocations. Translocations and deletions also can be identified in interphase or metaphase by using genomic probes that are derived from the breakpoints of recurring translocations or within the deleted segment.

Cytogenetics is particularly useful in the subclassification of acute myeloid leukemias and in confirming the diagnosis and prognosis of B-cell neoplasia. CLL, acute leukemias, B-cell lymphomas, and multiple myeloma may have cytogenetic abnormalities that can be detected using either conventional cytogenetics or FISH. An important application is use of FISH to identify the promyelocytic leukemia–retinoic acid receptor alpha (PML-RARA) translocation, t(15;17), in acute promyelocytic leukemia since FISH results are available before karyotype results. Although the sensitivity of FISH is higher at approximately 10^{-4} compared with a sensitivity of 10^{-2} for conventional cytogenetics, FISH requires the use of location-specific probes to identify specific aneuploidies or translocations, whereas conventional cytogenetics detects all chromosomal abnormalities if cells show mitotic activity. Rapid FISH assays may have turnaround times of only a few hours, although most standard FISH assays require 1 to 2 days. Conventional cytogenetics requires cells to grow, and thus the turnaround times vary from 4 up to 10 days.

Molecular diagnostics

PCR is designed to permit selective amplification of a specific target DNA sequence within total genomic DNA or a complex complementary DNA population. Partial DNA sequence information from the target sequences is required. Two oligonucleotide primers, which are specific

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Table 12-9 Common B-cell neoplasms

	CD20	CD5	CD10	CD23	CD43	cIg	sIg	Cyclin D1	Other
CLL/SLL	+	++	−	++	++	5%+	+	−	CD200+, CD79b+
LPL	++	−	−	−	+/−	+	+	−	
PLL	++	+/−		−			++	−	
HCL	++	−	−	−	−	−	+	+/−	CD11c+, CD25+, CD103+
MCL	++	++	−	−	++	−	++	++	CD200−
MZL	++	−	−	−	+/−	+/−	++	−	
FL	++	−	60% +	−/+	−	−	++	−	BCL2+, BCL6+
LCL	++	10%+	25%-50%+	−	+/−	+/−	+/−	−	BCL2+ in 30%-40%
BL	++	−	+	−	−	+	+	−	BCL2−
Myeloma	−/+	−	Occ +	−	+	++	−	15%-20%+	CD56+, CD38+, CD138+, MUM1+

BL, Burkitt lymphoma; cIg, cytoplasmic immunoglobulin; FL, follicular lymphoma; HCL, hairy cell leukemia; LCL, large-cell lymphoma; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; Occ, occasionally; MZL, marginal zone lymphoma; PLL, B-cell prolymphocytic leukemia; sIg, surface immunoglobulin; SLL, small lymphocytic lymphoma.

Table 12-10 Common mature T-cell and NK-cell neoplasms

	sCD3	cCD3	CD5	CD7	CD4	CD8	CD30	CD16	CD56	EBV	Other
T-PLL	+dim	+	+	+	+/−	−/+	−	−	+	−	TCL1+ CD52+
T−LGL	+	+	+	+	−	+	−	+	−	−	Granzyme+ TIA1+
NK leukemia	−	−	−	+/−	−	+/−	−	−	+	+	Granzyme+ TIA1+
EN-NK/T	−	+	−	+/−	−	−	−	+	+	+	Granzyme+ TIA1+
HSTL	+	+	−	+	−	−	−	+	+/−	−	Gamma delta TCR+ Alpha beta TCR-
Ent-T lym	+	+	+	+	−	+/−	+/−	−	−	−	Granzyme+ TIA1+ Perforin+ CD103+
SCPTL	+	+	+	+	−	+	+/−	−	−	−	Granzyme+ TIA1+ Perforin+ Alpha beta TCR+ CD123-
PTCL-NOS	+	−	+/−	+/−	+/−	+/−	+/−	−	+	+/−	
AILT	+	+	+	+	+/−	−	−	−	+	+/−	CD10+ CXCL13+ BCL6+ PD1+
ALCL	+	−	+/−	+/−	+/−	+/−	+	−	−	−	ALK+

AILT, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; cCD3, cytoplasmic CD3; EBV, Epstein-Barr virus; EN-NK/T, extranodal natural killer/T-cell lymphoma; Ent-T lym, enteropathy-associated T-cell lymphoma; HSTL, hepatosplenic T-cell lymphoma; NK leukemia, natural killer cell leukemia; PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified; sCD3, surface CD3; SCPTL, subcutaneous panniculitis-like T-cell lymphoma; T-LGL, T-cell large granular lymphocyte leukemia; T-PLL, T-prolymphocytic leukemia, TCR, T-cell receptor.

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Table 12-11 Immunohistochemical diagnosis of Hodgkin lymphoma

	CD45	CD30	CD15	CD20	CD3	PAX5
CHL (RS cells)	−	+	+	−	−	dim+
NLPHL (LP cells)	+	−	−	+	−	+
B-cell lymphoma	+	+/−	−	+	−	+
T-cell lymphoma	+	+/−	+/−	−	+	−

CHL, classic Hodgkin lymphoma; LP, lymphocyte predominant; NLPHL, nodular lymphocyte-predominant Hodgkin lymphoma; RS, Reed-Sternberg.

for the target sequence, are used. The primers are added to denatured single-stranded DNA. A heat-stable DNA polymerase and the 4 deoxynucleotide triphosphates are used to initiate the synthesis of new DNA strands. The newly synthesized DNA strands are used as templates for further cycles of amplification. The amplified DNA sequence can be detected via different methods; alternatively, the amplified DNA can be sequenced directly.

The many uses of PCR in clinical laboratories include detection of the break cluster region–Abelson tyrosine kinase (*BCR-ABL1*) translocation in chronic myeloid leukemia and detection of select genes, such as the Janus kinase-2 (*JAK2),* mutation in myeloproliferative neoplasms. PCR is appropriate for selected situations, including the rapid diagnosis of select mutations important in acute myeloid leukemia, such as the detection of *PML-RARA* in acute promyelocytic leukemia. PCR is also appropriate for detection of mutations in genes like FMS-like tyrosine kinase 3 (*FLT-3*) locus and calreticulin (*CALR*), which contain large indels, which may be missed by some mapping algorithms in massive parallel sequencing assays (next-generation sequencing [NGS]). Finally, PCR is used for standardized minimal residual disease testing, for example, *BCR-ABL1* in chronic myeloid leukemia.

DNA sequencing is important in the identification of point mutations. The earlier Sanger (chain termination) technique has been eclipsed by NGS technology (massive parallel sequencing), which has a high throughput capacity and thus makes parallel analysis of many genes possible. The clinical uses—including diagnosis, predictors of response to therapy, and risk stratification—are employed in a variety of hematologic malignancies, including myeloma, leukemias, and lymphoma, as well as identifying hereditary genetic mutations that predispose patients to inherited hematologic disorders. The tradeoff of the analysis of mutations in many genes is time, with most NGS panels requiring sophisticated bioinformatics pathways and curating of any detected variants that require 1 to 2 wk for results. In the United States, there are published recommendations for describing the significance of variants (mutations) detected with tier I through tier IV grading. Tier I variants indicate variants of strong clinical significance, whereas tier II variants are of potential clinical significance, tier III variants are of unknown clinical significance, and tier IV variants are benign or likely benign. Sanger sequencing is still used for select genes and to confirm some NGS results.

Miscellaneous laboratory hematology methods

Erythrocyte sedimentation rate

The erythrocyte sedimentation rate (ESR) measures a physical phenomenon—the opposing forces of gravity and buoyancy on RBCs when blood is suspended in an upright tube—and is expressed in millimeters per hour. Elevated plasma proteins, primarily fibrinogen and immunoglobulins, neutralize the RBC membrane negative charge, facilitating rouleau formation and more rapid sedimentation because of increased mass per surface area. The clinical utility of ESR generally is poor except for selected rheumatologic disorders, and it is not an appropriate screening test in asymptomatic patients. Conditions associated with elevated ESR include malignancies, infections, and inflammatory conditions (particularly polymyalgia rheumatica and temporal arteritis) as well as hematologic conditions, such as cold agglutinin disease, cryoglobulinemia, and plasma cell dyscrasia–related M proteins. ESR reference ranges increase with age and are higher for women. Additional variables affect ESR: anemia and macrocytosis can cause faster sedimentation, whereas sickle cells, by impeding formation of rouleaux and microcytosis, cause slower sedimentation. The modified Westergren method is the preferred manual method. Automated ESR analyzers monitor sedimentation for shorter periods, extrapolate to millimeters per hour, and correlate reasonably well with the Westergren method.

Solubility testing for hemoglobin S

Manual qualitative methods to detect hemoglobin (Hb) S rely on visual detection of turbidity when blood containing Hb S is added to a solution containing a

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reducing agent, detergent to lyse red blood cells, and high-concentration salt buffer. Deoxygenated Hb S forms tactoids that defract and reflect light, whereas nonsickling hemoglobins remain soluble, allowing the detection of lines or letters when viewed through the hemolysis solution. A positive solubility test cannot discriminate between Hb S trait, homozygous Hb S, Hb S/β-thalassemia, or other combinations that include Hb S. False-positive (FP) results can occur because of paraprotein or cryoglobulin precipitation, and false-negative (FN) results can occur in anemic (hemoglobin <7.0 g/dL) sickle trait individuals or when the Hb S concentration is <2.6 g/dL. Because the concentration of Hb S in affected newborns is low, sickle solubility testing should not be performed on infants <6 mo old because of the risk of FN results. If used as a screening test, a positive solubility test requires evaluation by an alternative method to confirm and quantify Hb S and to identify coexisting nonsickling hemoglobinopathies or thalassemias. Other rare hemoglobinopathies produce a positive solubility test, including Hb C Harlem, and if coinherited with Hb S, they produce a sickle cell disease phenotype.

Hemoglobin electrophoresis

Methods to separate normal (Hb A, A_2, and F) and abnormal hemoglobins, primarily based on differences in charge, include alkaline and acid gel electrophoresis, isoelectric focusing, high-performance liquid chromatography (HPLC), and capillary electrophoresis (Figure 12-3). No method can definitively identify and quantify all hemoglobin variants, and any abnormal hemoglobin identified by the method chosen for screening must be confirmed by an alternative method (including solubility test for presumed Hb S). HPLC and capillary electrophoresis analyzers are fully automated, provide precise measurements of normal and variant hemoglobins, and are well suited for laboratories performing many analyses to diagnosis hemoglobins S, C, and E as well as other uncommon hemoglobinopathies and β-thalassemia trait (elevated Hb A_2, microcytic anemia). For optimal genetic counseling, DNA analysis may be appropriate to completely characterize α-thalassemias and some uncommon thalassemias and hemoglobinopathies.

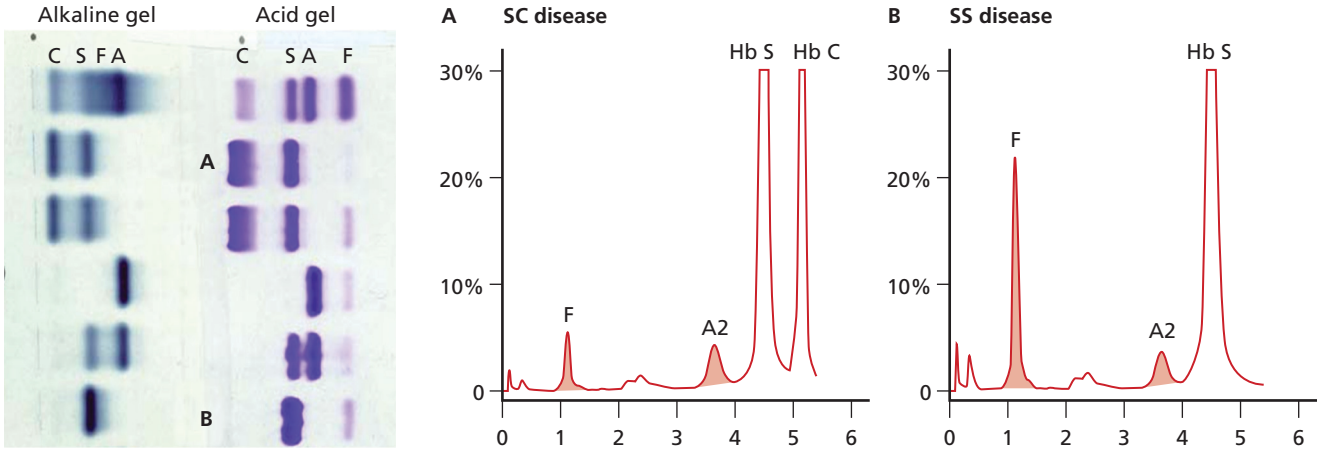
G6PD testing

Evaluation for inherited RBC enzymopathies is appropriate in patients with nonspherocytic, nonimmune-mediated hemolytic anemia (further discussed in Chapter 8). Sensitive qualitative screening tests for G6PD deficiency include dye decolorization and fluorescent spot tests, which monitor NADPH-dependent chemical reactions. FN results may occur if testing is performed during or shortly after a hemolytic event in individuals (typically African and African American males) with the A-mutation because enzyme activity is near normal in reticulocytes. Panels of additional RBC enzyme tests, such as pyruvate kinase testing, are offered by some laboratories.

Hereditary red cell skeletal disorders

The unique flexibility of an RBC depends on its lipid bilayer attachment to an underlying scaffold of α- and β-spectrin dimers via transmembrane proteins and other linking molecules. Inherited quantitative and qualitative RBC cytoskeleton defects are an infrequent cause of nonimmune chronic hemolysis, but these defects are relatively more common in people of northern European ancestry.

Figure 12-3 Examples of alkaline and acid gel electrophoresis and high-performance liquid chromatography patterns for a patient with hemoglobin SC disease (A) and a patient with homozygous sickle cell disease (Hb SS) (B). SC, compound heterozygous state for hemoglobin S and hemoglobin C.



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The most common phenotype is hereditary spherocytosis, see Chapter 8 for more details. Laboratory studies for HS can be performed to confirm loss of the RBC membrane, anchoring proteins, or spectrin. Although spherocytes are more susceptible to lysis when suspended in hypotonic saline solutions because of a decreased surface area or volume, increased osmotic fragility (OF) is an insensitive screening test for mild and compensated HS, and OF can produce FP results. A more sensitive and specific method is detection of decreased EMA binding by flow cytometry due to loss of RBC membrane proteins. Hereditary elliptocytosis causes minimal, if any, anemia and is a morphologic diagnosis (normal OF and EMA binding). Hereditary pyropoikilocytosis is caused by inheritance of both qualitative and quantitative RBC skeletal defects, which produce severe hemolytic anemia, deranged red cell morphologies, and decreased EMA.

Hemostasis testing

Hemostasis involves multiple molecular and cellular interactions to initiate and regulate platelet aggregation (primary hemostasis) and coagulation (secondary hemostasis) at the site of vascular injury to produce a durable "patch" without occluding blood flow. Laboratory evaluation of hemostasis is performed in several clinical settings, including screening of asymptomatic patients before selective invasive procedures and patients with underlying disorders associated with bleeding complications, evaluation of patients with personal or family histories of abnormal bleeding or bruising, assessment of inherited and acquired thrombotic risk factors, and anticoagulant drug monitoring.

Hemostasis screening typically consists of a prothrombin time (PT), activated partial thromboplastin time (aPTT), and platelet count. Abnormal screening tests require additional clinical and laboratory investigation to determine the underlying etiology. Mucosal bleeding, menorrhagia, petechiae, and ecchymoses suggest primary hemostatic disorders, such as von Willebrand disease (VWD) and qualitative platelet disorders, whereas hematomas, hemarthroses, and delayed bleeding suggest a coagulation factor defect.

Testing for thrombophilia is usually performed when a patient has a venous thromboembolic event (VTE) in the absence of compelling acquired risk factors, such as major surgery or trauma, cancer and its treatment, and immobility. The decision to test for a predisposition to VTE also depends on the patient's gender, age, history of thrombosis, and family history of thrombosis and whether the results would influence duration of anticoagulant therapy. Laboratory testing for inherited deficiencies of coagulation regulatory proteins should be done after a patient has completed treatment for a VTE and is in stable health. Levels of protein C (PC), protein S (PS), and antithrombin (AT) can be decreased because of consumption during the acute phase of a VTE and can also be reduced during anticoagulation treatment; PC and PS levels are reduced by warfarin, and AT levels are decreased during unfractionated heparin (UFH) therapy. In addition, the direct oral anticoagulants (DOACs) dabigatran, rivaroxaban, apixaban, and edoxaban can also interfere with thrombophilia testing. Lupus anticoagulant (LAC) testing should ideally be performed before anticoagulation is initiated in conjunction with serologic assays (anticardiolipin [aCL] and anti–β-2-glycoprotein I IgM and IgG antibodies), and abnormal results should be repeated at least 12 wk later to determine whether they are persistently abnormal to fulfill the laboratory classification criteria for antiphospholipid syndrome (APS). Genetic thrombophilia testing (factor V Leiden [FVL] and prothrombin 20210 gene mutations) can be ordered at any time and are unaffected by clinical status or medications. Heparin-induced thrombocytopenia (HIT) and TTP are unique acquired thrombocytopenia disorders with the potential for thrombotic complications. Laboratory test results can provide subsequent support for these diagnoses, but immediate therapeutic interventions should be based on clinical assessment in the absence of a rapid test.

Two major forms of anticoagulant therapy—warfarin antagonism of vitamin K–dependent γ-carboxylation of coagulation factors II, VII, IX, and X; proteins C and S; and UFH—require therapeutic drug monitoring because of unpredictable anticoagulant activities. Efforts to harmonize interlaboratory monitoring of warfarin with the PT ratio and heparin with the aPTT have led to the international normalized ratio (INR) and heparin activity (chromogenic anti-Xa) assays, respectively. The DOACs (dabigatran, rivaroxaban, apixaban, and edoxaban) do not require therapeutic drug monitoring; however, assays based upon anti-IIa or anti-Xa activity are available in some laboratories to measure drug concentrations in special clinical situations, such as bleeding, breakthrough thrombosis, suspected noncompliance, people with extremely increased or decreased body weight or body mass index, populations at risk for drug accumulation, and prior to urgent surgery or administration of thrombolytic therapy.

The following sections provide specific information regarding hemostasis laboratory methods as they apply to the aforementioned clinical situations.

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Preanalytical variables

Most laboratory errors occur in the preanalytical phase, which includes specimen collection, collection container composition and anticoagulant (tube type, see Table 12-1), tube fill volume and mixing, sample transport and processing, and duration and temperature of routine and frozen specimen storage. Samples sent for coagulation testing are especially susceptible to preanalytical variables.

For coagulation testing, the proportion of whole blood to sodium citrate anticoagulant volume is 9:1. Filling a tube with less than the recommended volume or collecting blood in the same proportions from a polycythemic patient (hematocrit >55%) increases the concentration of citrate in the plasma compartment, leading to incomplete recalcification when a fixed volume of $CaCl_2$ is added during test performance and resulting in artifactual prolongation of the PT or aPTT. Phlebotomy and coagulation services should follow guidelines for adjustment of the citrate volume in the blue top tube for patients with hematocrits >55%. There are no clear recommendations for adjusting citrate volume for anemic patients, as this has not been clearly demonstrated to impact clotting times.

Hemolysis, icterus, and lipemia/turbidity (HIL) in patient samples can also interfere with accurate measurement of coagulation assays. HIL can be attributable to in vitro processes resulting from incorrect sampling procedures, transport, or storage of specimens causing hemolyzed samples; in vivo RBC lysis (eg, from hereditary or acquired conditions, such as autoimmune hemolytic anemia, thrombotic microangiopathy, DIC) causing hemolysis; physicochemical mechanisms, such as the formation of chylomicrons and very-low-density lipoprotein after a high fat meal, administration of intravenous lipids, or an underlying metabolic disorder, such as diabetes, acute pancreatitis, or steroid administration causing sample lipemia/turbidity; and the presence of free (unconjugated) and direct (conjugated) bilirubin in icteric samples. Of the preanalytical errors in the coagulation laboratory, spurious hemolysis is the leading cause (19% to 40%), whereas icterus and lipemia are less common. HIL increases the spectrometric absorbance of the plasma sample and leads to high background absorbance readings, which may interfere with analyzers that use light-scattering clot detection methods, thereby compromising clot detection and accuracy of test results. Though analyzers may not be affected by or compensate for HIL, the quality of these specimens should be questioned because HIL can cause activation of coagulation.

In addition, exogenous interferences, such as presence of an anticoagulant or coagulation factor replacement therapy, may also interfere with plasma-based coagulation testing. Heparin contamination due to blood collection from central lines can cause a prolonged aPTT. A prolonged aPTT that corrects when repeated after treatment of plasma with a heparin-neutralizing agent confirms heparin contamination. Alternatively, a prolonged thrombin time (TT) and a normal reptilase time, which utilizes a snake venom not neutralized by heparin-accelerated AT, confirms the presence of heparin. Most PT reagents contain heparin-neutralizing agents, such as Polybrene, making this screening test relatively insensitive to heparin contamination. Many coagulation tests performed on plasma from patients taking oral direct factor IIa (dabigatran) and factor Xa (rivaroxaban, apixaban, edoxaban) anticoagulants are at risk for either positive or negative biases, which can be clinically important (Table 12-12). Current strategies to extend the half-life of FVIII and FIX concentrates include fusion to the Fc domain of human immunoglobulin, PEGylation, and albumin fusion, which can negatively impact recovery in 1-stage clotting assays depending upon the reagent used.

If a patient sample has an interference, the patient's test result may either be rejected or reported. If reported, the laboratory should annotate the result with a comment to indicate the presence and effect of the interference on the patient's result.

Screening coagulation testing and associated abnormalities

Most in vivo coagulation reactions are believed to be initiated by tissue factor activation of factor VII. Important interactions occur between the extrinsic and intrinsic pathways in physiologic in vivo hemostasis. Although the division into 2 separate pathways, as shown in Figure 12-4, does not reflect the complex interactions between coagulation factors in vivo, it does provide a useful way to interpret screening coagulation test results when evaluating for potential abnormalities of hemostasis.

Prothrombin time

The PT measures the time to form a fibrin clot after adding thromboplastin (source of tissue factor combined with phospholipid) and $CaCl_2$ to citrated plasma and assesses 3 of the 4 vitamin K–dependent factors (factors II, VII, and X) plus factor V and fibrinogen. Commercial thromboplastins contain either recombinant human tissue factor combined with phospholipid or thromboplastins derived from rabbit or bovine tissues. Almost all PT reagents contain a heparin-neutralizing additive to allow for monitoring of warfarin during concurrent heparin therapy.

Isolated prolongation of the PT most often reflects a deficiency of vitamin K–dependent factors resulting from

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Table 12-12 Coagulation tests interference caused by direct oral anticoagulants

Test	Factor IIa inhibitor (eg, dabigatran)	Factor Xa inhibitors (eg, rivaroxaban, apixaban)	Comments
aPCr ratio, aPTT based	+ bias	+ bias	Risk of missing FVL
Antithrombin, anti-Xa method	unaffected	+ bias	Risk of missing AT deficiency
Antithrombin, anti-IIa method	+ bias	unaffected	Risk of missing AT deficiency
Factors X,VII,V, II (PT based)	− bias	− bias	Possible inhibitor pattern
Factors PK, HMWK, XII, XI, IX,VIII (aPTT based)	− bias	− bias	Possible inhibitor pattern
LAC testing	abnormal	abnormal	Possible to misclassify as LAC
Protein C clotting assay	+ bias	+ bias	Risk of missing PC deficiency
Protein S clotting assay	+ bias	+ bias	Risk of missing PS deficiency
PT and aPTT	Prolonged	Prolonged*	
PT 1:1 mix	Prolonged	Prolonged	Inhibitor pattern
aPTT 1:1 mix	Prolonged	Prolonged	Inhibitor pattern
Thrombin time	Prolonged	Unaffected	
Fibrinogen acitivity (Clauss)	− bias with some methods†	Unaffected	
Chromogenic anti-Xa monitoring of heparin/LMWH	Unaffected	+ bias	Not a quantitative test for rivaroxaban, apixaban, edoxaban, or betrixaban unless calibrated with the specific drug

*Direct Xa inhibitors may have variable effects depending on the drug and drug concentration. In addition, different reagents show different sensitivities.

†Effect is method and drug dependent. Most fibrinogen assays show no effect with dabigatran. Bivalirudin can mildly decrease fibrinogen, whereas argatroban can significantly falsely reduce fibrinogen.

poor nutrition, inadequate absorption of vitamin K, antagonism of γ-carboxylation of the vitamin K–dependent factors by warfarin, or decreased hepatic synthesis. Causes of an isolated prolonged PT include preanalytical variables, congenital factor deficiencies (factor VII), acquired inhibitors, and anticoagulants (Figure 12-5).

Congenital deficiencies of factors X, V, and II and fibrinogen are rare (1 in 1 million to 2 million people), whereas the estimated prevalence of homozygous factor VII deficiency is 1 in 300,000 people. Some factor VII mutations produce greater PT prolongations with rabbit or bovine tissue factor than with human tissue factor. Therefore, it is important to confirm a suspected congenital factor VII deficiency by measuring factor VII activity with recombinant human thromboplastin. Dysfibrinogenemia occasionally causes a prolongation of the PT without a prolongation of the aPTT, and factor VII inhibitory autoantibodies are extremely rare.

Warfarin causes a prolonged PT and variably prolonged aPTT depending on the degree of factor IX, X, and II deficiencies. Therapeutic monitoring of warfarin depends on the PT. Thromboplastins, however, have different sensitivities to the effects of warfarin. To account for this variability and to obtain an international sensitivity index (ISI), reagent manufacturers compare PTs obtained with commercial thromboplastin lots to a World Health Organization reference thromboplastin with the behavior of recombinant or human tissue factor performed on plasma samples from healthy controls and stable, anticoagulated patients. A sensitive thromboplastin with an ISI of 1.0 is equivalent to human tissue, whereas a thromboplastin with an ISI of 2.0 is relatively insensitive to depletion of vitamin K–dependent clotting factors. The INR is the ratio of the patient's PT to the laboratory's PT geomean (average PT value derived from testing a group of normal individuals) raised to the exponent of the thromboplastin ISI ($INR = (Patient\ PT/Mean\ PT)^{ISI}$). The INR is designed to accurately monitor patients who have been stabilized on warfarin and allows comparisons between laboratories. It is *not* intended for assessing coagulopathies due to liver disease or DOACs because the ISI has not been validated for these conditions.

Activated partial thromboplastin time

The aPTT is a 2-step assay to measure the time to form a fibrin clot after incubation of citrated plasma with

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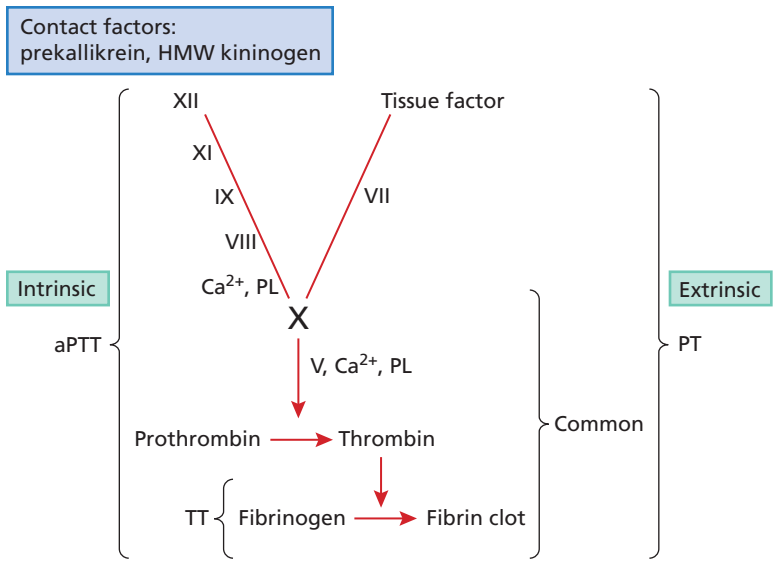


Figure 12-4 Simplified coagulation cascade indicating the intrinsic pathway measured by aPTT, the extrinsic pathway measured by PT, the common pathway (factor X, factor V, prothrombin, and fibrinogen) measured by PT and aPTT, and the conversion of fibrinogen to fibrin measured by TT and RT.

phospholipid and negatively charged particles followed by the addition of $CaCl_2$. The negative surface and phospholipid activate the contact factors (factor XII, prekallikrein [PK], and high-molecular-weight kininogen [HMWK]) and factor XI. The addition of $CaCl_2$ permits activation of factor IX and the remaining reactions to proceed to form a fibrin clot.

Causes of an isolated prolonged aPTT include preanalytical variables, congenital factor deficiencies of the intrinsic pathway, acquired inhibitors, and anticoagulants (Figure 12-6).

Deficiencies of factors VIII, IX, XI, and XII and PK and HMWK prolong the aPTT. Severe deficiencies of factor XII, PK, and HMWK are rare, typically produce

Figure 12-5 Algorithm for evaluation of an isolated prolonged PT.

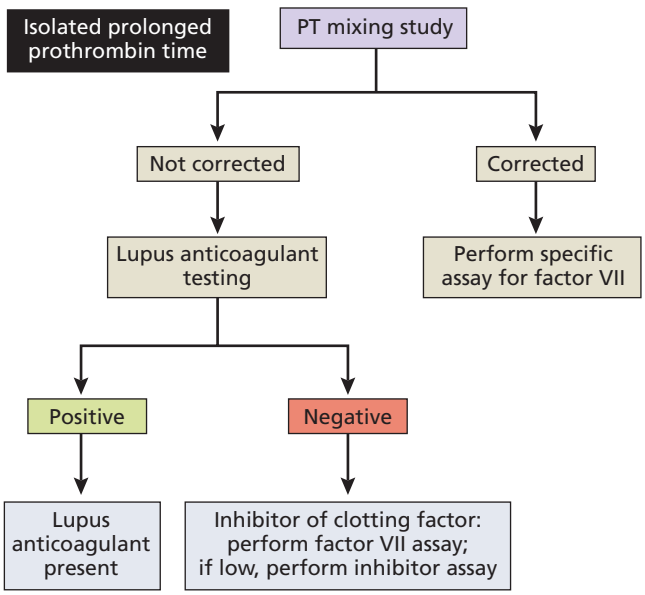
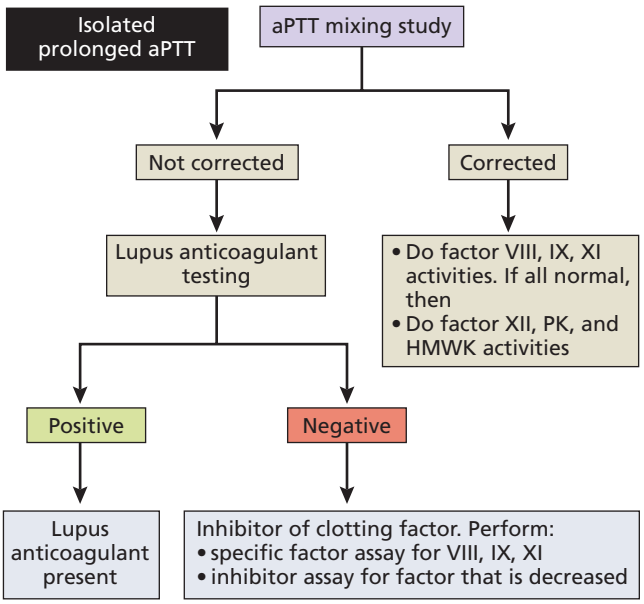


Figure 12-6 Algorithm for evaluation of an isolated prolonged aPTT.


Isolated prolonged aPTT
aPTT mixing study
Not corrected
Corrected
Lupus anticoagulant testing
• Do factor VIII, IX, XI activities. If all normal, then
• Do factor XII, PK, and HMWK activities
Positive
Negative
Lupus anticoagulant present
Inhibitor of clotting factor. Perform:
• specific factor assay for VIII, IX, XI
• inhibitor assay for factor that is decreased

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aPTTs >100 seconds and do not cause a bleeding disorder. Depending on the coagulation reagents and analyzer used for an isolated intrinsic factor deficiency to prolong the aPTT, factor activity is usually lower than 30% to 40% (or 30-40 IU/dL).

Factors VIII and IX deficiencies, or hemophilia A and B, respectively, are X-linked inherited disorders that often are diagnosed early in life because of spontaneous bleeding or a positive maternal family history of hemophilia. Occasionally, diagnosis is delayed until adulthood if it is a mild deficiency (5% to 40%).

Factor XI deficiency should be investigated when a prolonged aPTT is encountered in a person of Ashkenazi Jewish ancestry. Bleeding risk is variable and does not correlate particularly well with the severity of factor XI deficiency.

Patients with type 1 VWD may have a slightly prolonged aPTT if the factor VIII level is low, as von Willebrand factor (VWF) serves to stabilize FVIII. Patients with the type 2 N variant of VWD can have a moderate factor VIII deficiency, whereas patients with type 3 VWD typically have a severe deficiency of FVIII.

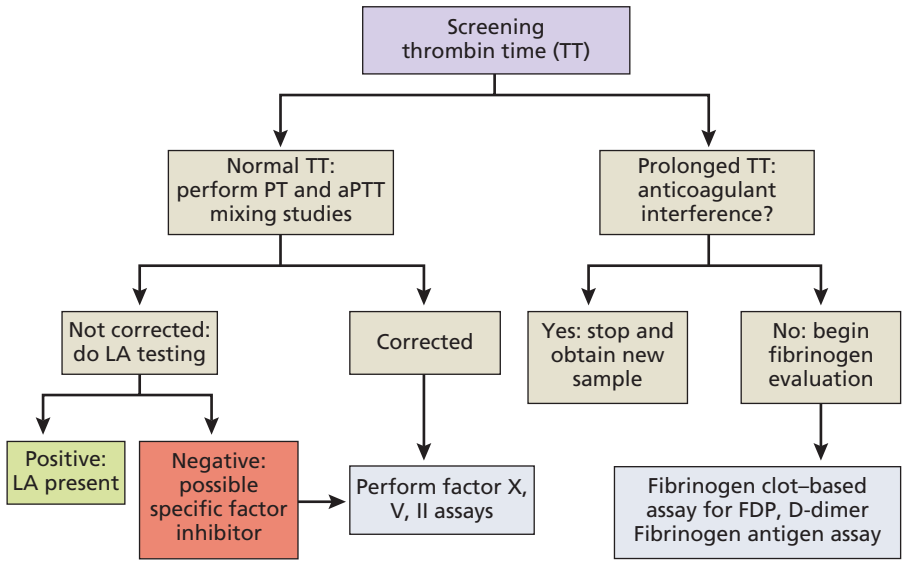
Lupus anticoagulants are immediate-acting in vitro anticoagulants that can cause a prolonged aPTT (see additional discussion in assays for thrombophilia). If a prolonged aPTT does not substantially shorten when repeated on a 1:1 mix with pooled normal plasma (PNP), LAC testing or specific factor activities should be performed depending on the clinical context.

Many hospitals still use aPTT-based nomograms to guide UFH anticoagulation. A therapeutic aPTT range is typically determined by collecting plasma samples from patients on heparin and comparing aPTTs with heparin activity using the chromogenic anti-Xa assay. The aPTT therapeutic range in seconds corresponds to an anti-Xa range of 0.3 to 0.7 IU/mL. However, the aPTT is an inferior yardstick for heparin level compared with direct measurement of ant Xa level. The aPTT is also used to monitor parenteral direct thrombin inhibitor therapy. For patients treated with bivalirudin, the therapeutic target recommended by the manufacturer is 1.5 to 2.5 times the baseline aPTT, whereas for argatroban the recommended target is 1.5 to 3.0 times the baseline aPTT. Therapeutic infusions of direct thrombin inhibitors may also prolong the PT/INR, and the intensity depends on the specific direct thrombin inhibitor and the thromboplastin reagent. The DOACs can prolong the aPTT and PT (Table 12-12); however, these assays cannot be used to predict plasma drug concentrations. As a result, assays based upon anti-IIa or anti-Xa are available in some laboratories to measure drug concentrations in special clinical situations, such as bleeding, breakthrough thrombosis, suspected noncompliance, populations at risk for drug accumulation, and prior to urgent surgery or administration of thrombolytic therapy.

Combined abnormalities of PT and aPTT

Deficiency or inhibition of a factor in the common pathway (factors X, V, and II and fibrinogen), multiple deficiencies, acquired dysfibrinogenemia, DIC, and LAC can cause combined prolongation of the PT and aPTT (Figure 12-7). Advanced liver disease can cause decreased hepatic synthesis of all coagulation factors except for

Figure 12-7 Algorithm for evaluation of a prolonged PT and aPTT. FDP, fibrin degradation product; TT, thrombin time.


Screening thrombin time (TT)
Normal TT: perform PT and aPTT mixing studies
Prolonged TT: anticoagulant interference?
Not corrected: do LA testing
Corrected
Yes: stop and obtain new sample
No: begin fibrinogen evaluation
Positive: LA present
Negative: possible specific factor inhibitor
Perform factor X, V, II assays
Fibrinogen clot–based assay for FDP, D-dimer Fibrinogen antigen assay

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factor VIII, and acquired dysfibrinogenemias are suggested by a low fibrinogen level in a functional assay combined with a normal or high level of immunologic fibrinogen (see the section "Fibrinogen assays" in this chapter).

Inhibitors to factor V can develop following exposure to bovine thrombin, which also contains bovine factor V, when combined with fibrinogen to produce "fibrin glue" during surgical procedures to control bleeding. Bovine factor V antibodies may crossreact with human factor V to cause bleeding in some patients. Low factor V activity and specific *in vitro* inhibition of factor V confirm the diagnosis. Fortunately, fibrin glue therapeutics containing either plasma-derived or recombinant human thrombin are now available.

Acquired prothrombin deficiency accompanies LACs rarely, causes moderately prolonged PTs, and can cause abnormal bleeding. The autoantibodies do not produce an inhibitor pattern in mixing studies because they are not directed against the active site of the molecule. Rather, they form immune complexes, increasing the clearance rate and lowering prothrombin activity.

PT and aPTT mixing studies

The purpose of a mixing study is to determine whether a prolonged aPTT or, occasionally, a prolonged PT is more likely because of a deficiency of 1 or more coagulation factors or to an inhibitor. The first step is to exclude contamination with heparin or other anticoagulant by performing a thrombin time (TT), heparin neutralization, or anti-Xa assay. Next, the aPTT or PT is repeated on a 1:1 mixture of patient plasma and PNP, which should provide at least 50% activity for all coagulation factors and substantial correction if a factor deficiency is the cause of a prolonged clotting time. Because factor VIII inhibitors and some LACs manifest their effect in prolonging the aPTT in a time- and temperature-dependent manner, 1:1 mixtures are incubated at 37°C for 1 to 2 h, which is followed by repeating the aPTT. There is no consensus approach for interpretation of mixing-study results, and inflexible requirements, such as correction to within the laboratory's PT or aPTT reference ranges to rule out inhibitor activity, can be misleading. One must consider the clinical context and the initial extent of PT and aPTT prolongation when assessing the 1:1 mix results. Sometimes mixing studies are not definitive, especially when an aPTT is mildly prolonged and corrects with mixing, in which case performing both selected factor activity assays and LAC screening may be necessary. Mixing studies may not be completely correct when there are multiple factor deficiencies as opposed to a single factor deficiency.

Coagulation factor activity assays

Determination of a specific coagulation factor activity in a patient's plasma typically is performed by 1-stage clotting assays performed on automated coagulation analyzers and requires 2 reagents: PNP and plasma deficient in the factor of interest. Combining equal volumes of plasma from a large number of healthy adults averages the interindividual variability for coagulation factors, which typically ranges from 50% to 150%, to produce PNP with 100% activity for all factors. Mixing PNP and factor-deficient plasma in different ratios produces calibrators of known factor activities, which is automated on most analyzers. PT-based assays are performed for factors VII, X, V, and II, and aPTT-based assays are performed for the intrinsic pathway factors. When the factor activities of the calibrators are plotted against the corresponding PT or aPTT results on logarithmic axes, a calibration line or curve is generated. Then, a PT or aPTT is performed on patient plasma mixed with factor-deficient plasma, and the corresponding activity is determined from the calibration curve.

Additionally, factor levels are determined at a minimum of 3 serial dilutions of a patient's plasma, and the results, corrected for the dilution factor, are compared. If an inhibitor is present, the factor activity appears to increase with dilution, and results are nonparallel to the calibration curve. To determine whether the inhibitor interference is specific for a factor, such as factor VIII, or nonspecific like an LAC may require performance of additional testing.

Current strategies to extend the half-life of FVIII and FIX concentrates include fusion of recombinant FVIII or FIX to the Fc domain of human immunoglobulin, PEGylation, and albumin fusion. These modifications have shown an improvement in half-life; however, they have also been shown to accentuate discrepancies with 1-stage clotting assays as compared with chromogenic assays. These differences are reagent specific, and laboratories should be aware of the sensitivity of their reagents to these modified products.

Factor VIII and IX chromogenic activity assays exist but are not widely available, with the exception of specialty laboratories and laboratories that support active hemophilia treatment programs. The end point of these assays is cleavage of a small peptide by an activated coagulation factor that generates a change in color (optical density) proportional to the activity of the factor. Chromogenic assays are more precise and demonstrate lower interlaboratory variability than 1-stage clotting assays. However, discrepant 1-stage clotting and chromogenic assay results exist, especially in patients with specific

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hemophilia A phenotypes or genetic mutations and when some recombinant factor concentrates are assayed.

Inhibitor assays

Inhibitors to factor VIII are detected in 25% to 30% of males with severe hemophilia A due to the development of alloantibodies to infusions of foreign factor VIII. Alloantibody formation to factor IX in males with severe hemophilia B occurs less often. Acquired hemophilia caused by autoantibodies to factor VIII is the most common acquired specific factor inhibitor. A factor VIII antibody is suspected in patients without a significant bleeding history who present with severe bleeding symptoms, and coagulation testing shows a prolonged aPTT that fails to fully correct immediately after 1:1 mixing and subsequently prolongs after a 1- to 2-hour incubation of the 1:1 mixture at 37°C. Very low or undetectable factor VIII activity and mild inhibitor patterns for factors IX and XI due to partial inhibition of factor VIII in these aPTT-based activity assays confirm the presence of a specific factor VIII inhibitor. The Bethesda assay determines the potency of a factor VIII inhibitor by incubating dilutions of patient plasma prepared with imidazole buffer combined 1:1 with PNP at 37°C for 2 h, which is followed by determination of residual factor VIII activity. Improvements to the traditional Bethesda assay include the Nijmegen modification or Nijmegen Bethesda assay, which incorporates buffered PNP and use of FVIII-deficient plasma instead of buffer for dilution and in the control. These assay modifications improve specificity for detection of low-titer inhibitors through reduction of nonspecific degradation of factor VIII during the 2-h incubation period. An additional modification of the assay (performing a heat treatment step of the patient sample at 56ºC for 30 min to eliminate infused or endogenous FVIII from the sample prior to testing) permits accurate testing in recently treated patients. The antibody titer is expressed in Bethesda units (BU) equal to the reciprocal of the patient plasma dilution required to obtain recovery of 50% of the expected factor VIII activity in the incubated 1:1 mixture. By definition, 1 BU is defined as the amount of inhibitor producing a residual factor VIII activity of 50%. A titer of 0.5 to 5.0 BU/mL is considered a low titer and may be overwhelmed with larger doses of factor VIII. However, a titer of >10 BU/mL requires treatment of bleeding episodes with alternative means, which might include use of recombinant porcine factor VIII for patients who do not have crossreactive antibody or a factor VIII–bypassing agent, such as either recombinant factor VIIa, activated prothrombin complex concentrate, or the more recently introduced monoclonal antibody emicizumab.

Fibrinogen assays

The Clauss method is a modified TT in which fibrinogen rather than thrombin is limiting. The time to clot formation is inversely proportional to fibrinogen activity calibrated against a standard of known concentration and expressed as milligrams per deciliter. The thrombin concentration usually is high enough to not be affected by therapeutic concentrations of heparin but can be affected by the presence of direct thrombin inhibitors. Fibrinogen activity can also be measured in a calibrated method based on the PT clotting time. Fibrinogen also can be measured in immunologic tests (fibrinogen antigen) to evaluate for possible dysfibrinogenemia (disproportionately low activity versus antigen). Routine fibrinogen testing in hospital labs is fibrinogen activity.

Thrombin time

The TT measures the time required to convert fibrinogen to a fibrin clot, bypassing the intrinsic, extrinsic, and common pathways. Achieving a normal TT requires sufficient amounts of normal fibrinogen and absence of thrombin inhibitors or substances that impede fibrin polymerization. The reagent is an excess of bovine or human thrombin, and the test sample is undiluted citrated plasma.

UFH, low-molecular-weight heparin (LMWH), argatroban, bivalirudin, and dabigatran inhibit the thrombin reagent and prolong the TT. Dysfibrinogenemias usually prolong the TT and are suspected if the functional test (fibrinogen activity) is disproportionately low compared with an immunologic measurement of fibrinogen (fibrinogen antigen). Hypofibrinogenemia usually prolongs the TT when levels of fibrinogen are below approximately 90 mg/dL. L-asparaginase can cause hypofibrinogenemia by inhibiting synthesis. Fibrin degradation products in very high concentrations and M proteins can inhibit fibrin polymerization and prolong the TT. Heparin-like anticoagulants (heparan sulfates) have occurred in patients with multiple myeloma and other tumors; they prolong the TT by interacting with AT in a manner similar to heparin, but the reptilase time is normal in these patients.

Reptilase time

Reptilase is snake venom that cleaves only fibrinopeptide A from fibrinogen (in contrast to thrombin, which cleaves both fibrinopeptide A and fibrinopeptide B) and results in fibrin clot formation. This assay is prolonged by hypofibrinogenemia and most dysfibrinogenemias but is not prolonged by heparin because the reptilase enzyme is not inactivated by AT or direct thrombin inhibitors.

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Global hemostasis tests

Thromboelastography/thromboelastometry involves monitoring the viscoelastic properties of whole blood during clot initiation, stabilization, and lysis. Two commercial instruments: TEG (Haemonetics, Braintree, MA) and ROTEM (Instrumentation Laboratory, Bedford MA) are currently available in most geographies. The change in viscosity of blood as it clots in a cup is transmitted through a pin immersed into the blood through a mechanical-electrical transducer, producing a tracing of clot firmness over time. The laboratory reports parameters indicating time to initiation of clot formation, the rate of initial clot formation, the maximum clot firmness, and a measure reflecting the degradation of clot firmness over time. Certain patterns correlate with coagulopathies, hypofibrinogenemia, thrombocytopenia, and hyperfibrinolysis. Most experience with viscoelastic testing has been in liver transplantation, trauma, and cardiopulmonary bypass surgical settings, in which rapid point-of-care hemostasis information is used to select appropriate blood component transfusion and factor replacement therapy.

Point-of-care hemostasis tests

There are a number of commercially available point-of-care (POC) coagulation devices that utilize whole-blood samples and measure PT/INR, aPTT, and activated clotting time (ACT). These devices vary with regard to specimen volume requirements, active reagents, and endpoint detection methods but have in common single-use test cartridges.

With the growing numbers of anticoagulation clinics and anticoagulation management services, patient self-testing and patient self-management with POC PT/INR testing has increased. However, POC devices that determine a thromboplastin-initiated clotting time that is electronically converted to a PT and INR have limitations in accuracy and precision when compared with laboratory-based methods. These limitations include incorrect calibration of the ISI to the World Health Organization standard, extrapolated mean normal PT, and nonlinearity at supratherapeutic levels. Although the evidence does not support widespread use of POC INR testing in general practice, patient self-testing and patient self-management have been associated with improved anticoagulation control and decreased incidence of thromboembolic or major bleeding events.

UFH levels greater than 1 unit/mL may infinitely prolong the aPTT. As a result, the ACT, which measures the time in seconds from the activation of factor XII to the formation of a fibrin clot, remains the predominant test to manage UFH anticoagulation during interventional cardiac and vascular procedures and during cardiopulmonary bypass. The test is performed in whole blood rather than plasma, which decreases heparin sensitivity and allows clotting time results even when high concentrations of heparin are present. ACT assays use different activators (celite, kaolin) and are optimized for specific heparin ranges, from low-dose heparin concentrations, such as those used in extracorporeal life support, to high-dose heparin therapy used in cardiac surgery. In addition, the ACT is impacted by other factors, including thrombocytopenia, platelet dysfunction, hemodilution, hypofibrinogenemia, coagulation factor deficiencies, LACs, and hypothermia.

von Willebrand factor assays

Endothelial cells and megakaryocytes synthesize VWF, which plays an important role in platelet adhesion and stabilization of factor VIII. von Willebrand disease is due to inherited quantitative or qualitative defects of VWF. Acquired defects may also result in a bleeding disorder termed acquired von Willebrand syndrome. Laboratory testing for suspected VWD is challenging because of the variability of personal and family bleeding histories, multiple types of VWF defects, physiologic variables affecting VWF levels, and analytical imprecision of certain VWF test methods. Repeated testing is indicated to confirm abnormal results before diagnosing a patient with VWD. See Chapter 10 for additional information regarding clinical presentation, classification, and management of VWD.

Initial testing for von Willebrand disease

Global tests of primary hemostasis, including bleeding time (which is rarely performed today) and PFA-100/200 closure times, lack both sensitivity and specificity for VWD, and the aPTT is an indirect and potentially insensitive screening test for low factor VIII activity. VWF antigen concentration (VWF:Ag), VWF platelet-binding activity, VWF collagen-binding activity, and factor VIII activity measurements are sufficient initial screening tests. VWF levels vary based on blood type, with type O individuals having mean values approximately 25% lower than non–type O controls. Blood type–specific reference intervals for asymptomatic type O individuals may be as low as 35% to 40%. However, it is reasonable to consider VWF levels in the range of 30% to 50% as a risk factor for a mild bleeding tendency, and such mild VWF deficiency is not necessarily as an indication of an inheritable disease. Fluctuations of VWF in patients during physiologic alterations associated with acute stresses, the menstrual cycle, or pregnancy make the interpretation of these analytes problematic,

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and patients may require repeat testing. Several equivalent and accurate methods can be used to quantify VWF:Ag. Measuring VWF platelet-binding activity is another matter. The most widely used method and the current gold standard is the ristocetin cofactor assay (VWF:RCo), which can be performed by automated immunoturbidity assays using lyophilized platelets and ristocetin or by platelet aggregometry and assesses VWF binding to the platelet GPIb/IX/V complex. Ristocetin, an antibiotic, binds to VWF, causing a conformational change that mimics the effect of high shear stress in vivo to expose the platelet-binding domain. The VWF:RCo activity is sensitive to both quantitative deficiencies of VWF (type 1 and type 3 deficiencies) and to mutations causing reductions in high- and intermediate-weight VWF multimers or defects in platelet binding (types 2A, 2B, and 2M VWD). It is also mildly reduced in patients with the benign VWF amino acid polymorphism Asp1472His (D1472H) that occurs in the region where ristocetin interacts with VWF, a polymorphism that is common, especially in people of African American heritage. VWF:RCo/VWF:Ag ratio of <0.7 suggests a qualitative or type 2 VWF defect and warrants specialized confirmatory testing (Table 12-13; see video in online edition). The VWF:RCo assay is labor intensive, poorly standardized, and imprecise, leading to the development of alternative methods to assess VWF platelet-binding activity, including ristocetin-induced binding to recombinant wild-type GPIb fragments (VWF:GPIbR), spontaneous binding (no ristocetin requirement) of VWF to a gain-of-function mutant GPIb fragment (VWF:GPIbM), or by binding of a monoclonal antibody to a VWF A1 domain epitope (VWF:Ab). Moreover, ristocetin binding site polymorphisms have been described and may affect the measurement of VWF activity by VWF:RCo. Although the newer alternative methods may have improved technical performance characteristics, there is currently limited availability and regulatory approval for these assays in some locations.

Specialized testing to classify von Willebrand disease

Dismissing a diagnosis of VWD or confirming a diagnosis of type 1 or type 3 VWD usually can be accomplished by reviewing VWF:Ag, VWF:RCo, and factor VIII activity results. A VWF:RCo or factor VIII activity result much lower than VWF:Ag (ratio <0.7) is an indication for more specific testing.

VWF multimer analysis provides qualitative information by identifying structural abnormalities that correlate with qualitative defects in VWF adhesion (Figure 12-8). Electrophoresis of plasma through low-concentration agarose gel separates VWF multimer bands by size, which are detected with radiolabeled, enzyme-linked, or fluorescent VWF antibodies. Analysis of the band patterns can distinguish normal or subtly abnormal patterns (consistent with type 1 and 2N or 2M VWD, respectively) from major losses of high- and intermediate-size bands (consistent with type 2A, type 2B, and platelet-type VWD).

The ristocetin-induced platelet aggregation assay is a variation on the VWF:RCo assay to investigate platelet adhesion defects. Several ristocetin concentrations (ranging from 0.5 to 1.5 mg/mL) are added to separate aliquots of a patient's platelet-rich plasma. A change in light transmission is monitored by an aggregometer as platelets bind to VWF and aggregate (Figure 12-9). Normal and mild type 1 VWD platelet-rich plasma typically produces no or minimal aggregation at low ristocetin concentrations and increasing aggregation at higher concentrations. Platelet-rich plasma from severe type 1 and types 2A and 2M VWD patients may produce attenuated aggregation at high ristocetin concentrations due to VWF deficiency, whereas platelet-rich plasma from type 2B or platelet-type VWD patients shows an enhanced aggregation response

Table 12-13 Assays for VWD classification

VWD type	VWF activity	VWF antigen	Ratio of VWF activity to antigen	RIPA	FVIII	Multimers
Type 1	↓	↓	≥0.7	Normal to ↓	Normal to ↓	Normal pattern
Type 2A	↓↓	↓	<0.7	Normal to ↓	Normal to ↓	↓ High
Type 2B	↓↓	↓	<0.7	↑↑↑	Normal to ↓	↓ High
Type 2M	↓↓	↓	<0.7	↓↓	Normal to ↓	Normal pattern
Type 2N	Normal to ↓	Normal to ↓	≥0.7	Normal	↓↓	Normal pattern
Type 3	↓↓↓	↓↓↓	N/A	↓↓↓	↓↓↓	Undetectable

VWF activity indicates platelet-binding activity (VWF:RCo, VWF:GPIbM, or VWF:GPIbR).

N/A, not applicable; RIPA, ristocetin-induced platelet aggregation.

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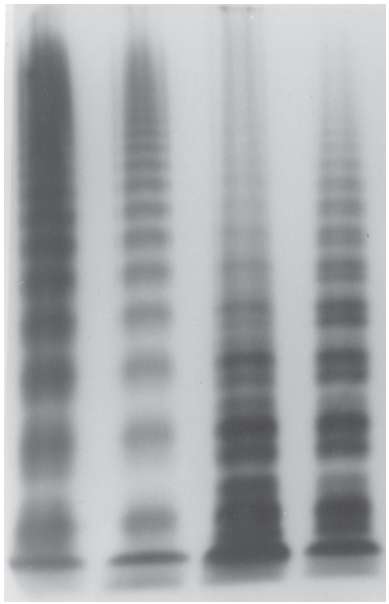

Figure 12-8 von Willebrand multimer patterns. 1, type 1 VWD with normal bands but decreased staining intensity; 2A, type 2A VWD with loss of high and intermediate multimers; 2B, type 2B VWD with loss of high-molecular-weight multimers; NP, normal plasma.

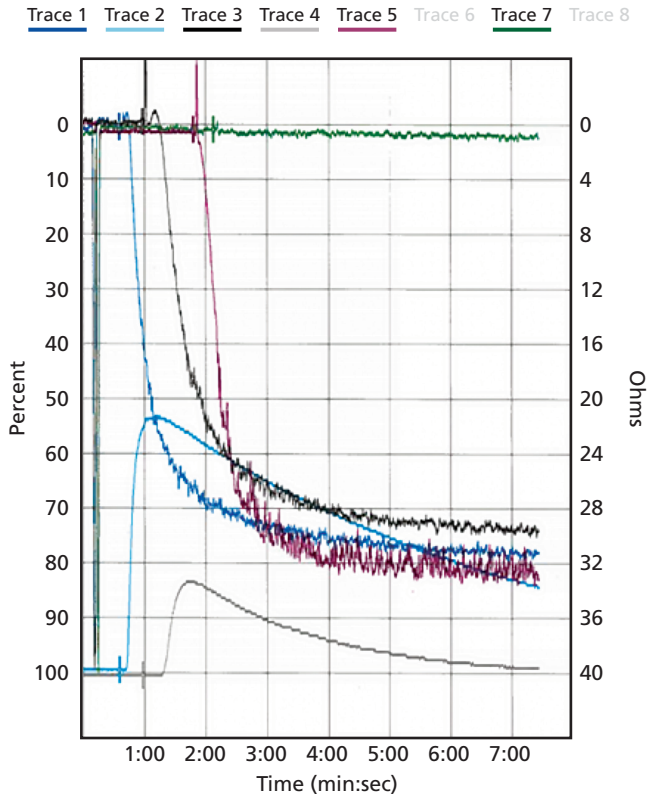

Figure 12-9 Representative aggregation and lumi-aggregometry curves performed on normal platelet-rich plasma. Tracings coming down from the upper baseline indicate aggregation response and tracings rising from the lower baseline indicate ATP release from dense granules. Blue: thrombin receptor activation peptide; black: lines arachidonic acid, 0.5 mg/mL; red: high-dose ristocetin, 1.5 mg/mL; green: low-dose ristocetin, 0.5 mg/mL.

to low ristocetin concentrations. Estimates of the relative frequency of type 2B VWD to platelet-type VWD range from 8:1 to 10:1. Although the disorders have similar clinical presentations and inheritance is autosomal dominant, they require different types of hemostasis replacement products (VWF concentrate versus platelet transfusion, respectively). Mixing studies using normal washed platelets plus patient plasma or vice versa can distinguish whether the patient's VWF or platelet receptor is abnormal; however, such procedures are difficult to perform, are not widely available, and may lack diagnostic specificity. Some reference laboratories perform platelet-VWF binding assays using a VWF monoclonal antibody to assess the ability of a patient's VWF to bind formalin-fixed platelets in the presence of low-dose ristocetin. Genetic testing to detect known mutations associated with each subtype and targeted sequence analysis (*VWF* exon 28 for types 2B and 2M; *GP1BA* for platelet-type VWD) are offered by a few reference laboratories.

Rarely, men and women with mild or moderate factor VIII deficiencies have that deficiency because of reduced VWF ability to chaperone factor VIII effectively. Such patients lack a familial X-linked inheritance pattern consistent with hemophilia A, demonstrate suboptimal response to factor VIII concentrates, and may be either genetically homozygous for type 2N VWD (decreased VWF binding affinity for factor VIII) or compound heterozygous (type 1/2N). Decreased binding of recombinant factor VIII to the patient's immobilized VWF in an enzyme-linked immunosorbent assay (ELISA) and equivalent VWF:Ag and VWF platelet-binding activity results are consistent with type 2N VWD. Genotyping specific for type 2N mutations is offered by a few reference laboratories and can differentiate hemophilia A or hemophilia A carriers from 2N VWD in difficult cases. Aside from targeted sequencing for type 2 VWD subtypes, a few laboratories offer whole-gene VWF sequencing. This is not commonly used but allows identification of genetic abnormalities throughout the large VWF gene.

VWF propeptide (VWFpp) is cleaved from mature VWF, and these are secreted in equimolar amounts. The ratio between VWFpp and VWF:Ag can help to assess VWF synthesis, secretion, and clearance rates of VWF.

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VWD with decreased VWF half-life due to increased clearance of mutant protein, as seen in some cases of type 1 VWD, demonstrate an increased ratio. However, current guidelines recommend using a desmopressin trial with 1- and 4-h postinfusion testing to identify cases with increased clearance rather than use of propeptide to antigen measurements.

Bleeding disorders with normal screening hemostasis tests

Abnormal, typically delayed bleeding due to severe factor XIII deficiency or fibrinolytic pathway defects are rare yet should be considered when evaluations for coagulopathies and primary hemostasis defects are negative. Thrombin activates factor XIII, and factor XIIIa crosslinks fibrin monomers to produce a durable clot. The urea clot solubility test is a qualitative screening test for severe factor XIII deficiency. Thrombin is added to plasma, and the clotted fibrin is added to a high-molar solution of urea that disrupts the clot if fibrin has not been crosslinked by factor XIIIa. This qualitative assay is only abnormal in patients with more severe factor XIII deficiency in whom the factor XIII activity is <5%. Alternative quantitative assays are available to directly quantify factor XIII antigen and activity; such assays identify both milder and more severe deficiency states.

Global screening tests of the fibrinolytic system include the euglobulin clot lysis time, which measures the time to lyse a fibrin clot in the absence of plasmin inhibitors, and the whole-blood clot lysis time (see the section "Global hemostasis tests" in this chapter). Congenital hyperfibrinolysis is due to deficiencies of natural plasmin inhibitors, plasminogen activator inhibitor 1 (PAI-1), and α_2-antiplasmin, and laboratory evaluation requires a panel of analytes, including plasminogen, PAI-1 activity and antigen, tissue plasminogen activator (tPA) antigen, and α_2-antiplasmin activity typically performed in reference laboratories. Causes of acquired hyperfibrinolysis resulting in circulating plasmin overwhelming α_2-antiplasmin inhibition include decreased hepatic clearance of tPA due to advanced cirrhosis or during liver transplantation, increased release of tPA from endothelial cells during cardiopulmonary bypass, amyloidosis, envenomization from several species of snakes, and as a component of DIC associated with acute promyelocytic leukemia and rarely with solid tumors, including bladder or prostate cancer. Laboratory evidence for primary fibrinolysis includes reduced fibrinogen levels due to cleavage by plasmin, elevated fibrin(ogen) degradation products, and no significant elevation of D-dimer levels because lysis of crosslinked fibrin clot is not the dominant process. DIC is the result of a primary disease process that leads to the release of tissue factor or other coagulation-activating factors into the blood. Because of variations in the amount and rate of procoagulant material released varies by the underlying disease, there is no specific diagnostic pattern of laboratory results. In acute, overwhelming DIC, initial platelet counts and fibrinogen levels are low, or serial testing shows a downward trend. PT, aPTT, and TT may be prolonged depending on the severity of consumption, and D-dimer levels are markedly elevated, indicating unregulated thrombin activity and secondary fibrinolysis. The International Society on Thrombosis and Haemostasis has developed a scoring system for evaluation of lab studies in a patient for whom the diagnosis of DIC is being considered, which includes platelet count, markers of fibrin degradation, prothrombin time, and fibrinogen level. Prospective studies suggest a sensitivity of 93% and specificity as high as 98%, and scores appeared to correlate with prognosis in patients with sepsis.

Vessel wall defects, such as collagen diseases (eg, Ehlers-Danlos and Marfan syndromes), also can cause abnormal bleeding. In addition to physical examination and imaging information, genetic testing is becoming more readily available for some of these syndromes.

Heparin monitoring

Some hospitals still use aPTT-based nomograms to guide UFH anticoagulation; however, monitoring heparin anticoagulation with the chromogenic anti-Xa assay is the preferred approach in many hospitals as an alternative to aPTT. Advantages of using anti-Xa for UFH monitoring include a shorter time to a therapeutic result; less variability resulting in decreased dosage changes and ordered tests; no confounding from factor deficiencies, LACs, or acute phase reactants; and limited interferences from common biologic substances. In addition, anti-Xa can be used when a patient's baseline aPTT is prolonged because of an LAC or deficiency of a contact activator (XII, PK, or HMWK).

The anti-Xa assay is a variation of a chromogenic AT assay (see section on assays for thrombophilia) comparing an unknown concentration of heparin in the patient plasma with a calibration curve prepared with a UFH, LMWH, or hybrid curve. Following addition of activated factor Xa to the test plasma, the rate of factor Xa neutralization by AT is positively correlated with the heparin concentration, and the rate of chromogenic substrate cleavage by factor Xa is inversely correlated with the heparin concentration.

LMWHs may minimally prolong the aPTT at therapeutic concentrations. Therapy with LMWHs typically

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does not require monitoring. However, under certain situations, including patients of extremely low and high body weight, pediatric patients, pregnant patients, and patients with impaired renal function, monitoring peak plasma LMWH activity (approximately 4 h after a subcutaneous injection) using a chromogenic anti-Xa assay is recommended.

Platelet function tests

The diagnosis of platelet disorders is covered in Chapter 11. Evaluation begins with determination of platelet count and platelet size as reported by automated analyzers and evaluation of platelet morphology on a Wright-stained slide. In vitro assessment of platelet activation and aggregation in response to selected platelet agonists should be reserved for patients with convincing bleeding histories in whom evaluations for coagulopathies, VWD, and moderate-to-severe thrombocytopenia are negative. Many disease processes can produce acquired qualitative platelet defects, including uremia, liver failure, and myeloproliferative and myelodysplastic disorders, but formal aggregation studies are usually not informative in these cases. Platelet function testing is technically demanding, time consuming, and poorly standardized, even despite recent guidelines for performing and interpreting these studies. The hematologist should be aware that labs use different platforms to analyze platelet aggregation: instruments that are used to test platelet-rich plasma (light transmission aggregometry) and instruments that use whole blood (whole-blood aggregometry). Testing is performed on aliquots of citrated whole blood or platelet-rich plasma with different concentrations of agonists, such as adenosine diphosphate (ADP), epinephrine, and collagen; arachidonic acid, which platelets metabolize to the agonist thromboxane A_2 via the cyclooxygenase pathway; and ristocetin to screen for platelet GPIb/IX/V deficiency (ristocetin response is also abnormal in moderate-to-severe VWD). Formation of platelet aggregates causes an increase in light transmission over time for assays using platelet-rich plasma and increased electrical resistance between electrodes in whole-blood aggregation assays. The platelet release reaction can be assessed in a lumi-aggregometer, which simultaneously monitors whole-blood aggregometry through changes in electrical impedance as platelets aggregate and platelet activation when released adenosine triphosphate (ATP) combines with luciferin/luciferase enzyme-releasing light. Figure 12-9 shows normal aggregation and release responses. Certain patterns of platelet aggregation responses to a panel of agonists are sensitive to specific inherited and rare qualitative platelet disorders, including Glanzmann thrombasthenia, Bernard-Soulier syndrome, and collagen receptor defects. Platelet secretion defects resulting from abnormal signal transduction and qualitative and quantitative granule disorders are more common, produce variable aggregation patterns, and require additional diagnostic tests that are not readily available for clinical use. As with the PFA-100 assay (described in the following), several preanalytic variables may affect aggregation results. Prescribed and over-the-counter medications that can inhibit platelet function must be discontinued before testing. Supplemental studies, including whole mount platelet electron microscopy to count dense granules or transmission electron microscopy to evaluate finer cell structures, may be accessible through research or reference laboratories. Finally, the list of genes responsible for either congenital thrombocytopenia or platelet function defects continues to grow, and genetic sequence analysis may be considered to confirm a specific diagnosis or probe further for a patient in whom inherited platelet disorder is suspected.

Global primary hemostasis screening tests

The template bleeding time is an invasive test fraught with difficult-to-control technical and patient variables and lacks sensitivity and specificity for detection of primary hemostasis disorders. Prolonged bleeding times performed on asymptomatic patients do not predict a risk of abnormal bleeding during surgery or other invasive procedures. The test is performed by making a standard incision in the forearm using a spring-loaded blade while maintaining a blood pressure cuff at 40 mm Hg. Blood oozing from the incision is wicked away with filter paper every 30 s until bleeding stops. The typical reference range in adults is approximately 5 to 10 min.

Most laboratories have discontinued performing bleeding time tests and substituted automated in vitro screening methods, which do not require an incision and provide more precise results from samples of blood collected in citrate yet have similar limitations. The PFA-100/200 instrument monitors VWF-dependent platelet adhesion and aggregation under conditions that mimic the shear forces in the arterial circulation. Citrated blood is aspirated through a metal tube to expose blood components to shear forces and then through a minute aperture in a membrane coated with collagen (COLL) and ADP (COLL/ADP) or collagen and epinephrine (COLL/EPI). VWF multimers bind to collagen, and platelets adhere to VWF, are activated by COLL/ADP or COLL/EPI, aggregate, and occlude the aperture, which is recorded as closure time in seconds. Each laboratory must determine reference intervals, although typical ranges are 55 to 137 s and 78 to 199 s for COLL/ADP and COLL/EPI cartridges, respectively. Prolonged PFA-100/200 closure times are not sufficiently sensitive for all congenital qualitative platelet

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disorders and types of VWD to be used as a general screening test. In addition, as anemia and thrombocytopenia worsen, closure times increase, and these variables should be considered when interpreting prolonged closure times in the setting of hematocrit <30% and platelet count $<100 \times 10^6$/mL. Prolonged COLL/EPI closure time may be a sensitive test for aspirin inhibition of platelets, but the COLL/ADP closure time is insensitive to blockade of the platelet P2Y12 ADP receptor by thienopyridines.

Specialized testing for acquired thrombocytopenia

Assays for platelet antibodies

Immune-mediated thrombocytopenia remains a clinical diagnosis of exclusion due to the general poor performance of laboratory methods to detect platelet-specific antibodies. Assays detecting total or surface-bound platelet immunoglobulins are nonspecific and are not recommended.

Assays for heparin-induced thrombocytopenia

HIT is a "clinical-pathological syndrome" in which diagnosis is supported by specific clinical parameters in combination with serologic and functional assays. The disease is driven by IgG antibodies directed against platelet factor 4 (PF4)/heparin complexes. The typical features include fall in platelet count by more than 50% or new thromboembolic complications generally occurring 5 to 10 d after heparin exposure. Two different types of lab assays are deployed in evaluation of a patient with suspected HIT: immunoassays that detect anti-PF4/heparin antibodies and functional assays that detect a subgroup of pathogenic heparin-dependent platelet-activating antibodies.

Commercial enzyme-linked immunosorbent assays (eg, ELISA) detect antibodies recognizing immobilized PF4 bound to heparin or polyvinyl sulfonate complex (heparin substitute). Although highly sensitive, HIT ELISA results are nonspecific, detecting both antibodies capable of activating platelets in vivo driving thrombocytopenia and thrombosis in the patient as well as benign antibodies that are not platelet activating. ELISA is highly sensitive, with as many as 97% of HIT patients having a positive ELISA, However, the positive predictive value of a positive PF4 ELISA result alone to confirm a diagnosis of HIT is low, and if used as the only criterion, a positive PF4 ELISA results in the overdiagnosis of HIT. Several approaches are available to improve the specificity of PF4 ELISA testing. First, clinicians can improve the pretest likelihood that thrombocytopenia is due to HIT by applying a validated clinical scoring system, such as the 4Ts (thrombocytopenia, timing, thrombosis, and exclusion of other more likely causes of thrombocytopenia). Patients with low 4T scores (≤3) are unlikely to have HIT, even with a positive PF4 ELISA, removing the need for testing. This is especially true for patients who have an increased likelihood of having an FP test, such as patients who have recently had cardiopulmonary bypass. Only IgG class anti-PF4/heparin immunoglobulin appears to be platelet activating; ELISA that selectively detects IgG has improved specificity with little impact on sensitivity. Finally, ample evidence suggests that the higher an HIT ELISA optical density (OD) is, the more likely it is that a functional HIT assay will be positive and that the patient's clinical presentation and response to therapy will be consistent with HIT. Conversion from viewing HIT ELISA results as simply positive or negative to considering OD as a continuous variable, with increasing probability for HIT as OD increases, is still evolving as clinical research continues.

In vitro functional assays that report activation of control platelets by patient serum in the presence of therapeutic concentrations of heparin are considered the "gold standard" for laboratory corroboration of HIT. Activation with a low/therapeutic heparin concentration and no activation at high heparin concentration is considered to be both specific and sensitive for detection of PF4 heparin-immune complexes, which are capable of causing in vivo platelet activation, thrombocytopenia, and thrombosis. In North America, selective laboratories perform the serotonin release assay (SRA) to monitor serotonin secretion from control platelets. Both the assay sensitivity and specificity are high (>88%). In Europe, heparin-induced platelet-activation assay (HIPAA) performed in microtiter wells with visual detection of platelet aggregation is the preferred method. Both assays are technically difficult, labor intensive, and not readily available. As a consequence, clinicians are often left to make management decisions based upon clinical judgment and ELISA data while awaiting the results of activation assay from a reference laboratory.

Several advances in laboratory assays have recently materialized. For rapid detection of PF4/heparin antibodies, automated latex agglutination assay and chemiluminescent immunoassays have been added to the armamentarium of HIT testing. The latex agglutination assay is considered a "functionalized immunoassay" as it detects HIT antibodies based upon their ability to inhibit agglutination of latex-coated particles coated with the HIT-like monoclonal antibody KKO following the addition of PF4/polyvinylsulfonate complex. In a single institution prospective cohort study of 4Ts scoring and consecutive HIT patients using reference SRA for diagnostic classification, the authors demonstrated high sensitivity (>94%) and high specificity (97%) with a positive

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predictive value of 56% with negative predictive value of 99%. Similar evaluation by 2 centers of the chemiluminescent assay showed a high combination of sensitivity (85%-97%) and specificity (93%-98%). The combination of commercially available lab automation, short analytical turnaround times, and on-demand availability of accurate assays may eventually allow clinicians to make an informed decision before switching to alternative anticoagulation. Advances are also being reported related to platelet-activation assays to confirm a diagnosis of HIT. Although still a technically demanding assay, the PF4-dependent P-selectin expression assay appears to have similar sensitivity and specificity to the SRA, and because that assay does not require a radioactive label (some SRA methods use radiolabeled serotonin), it is possible that such assay could improve the availability of a platelet-activation assay.

Assays for evaluation of thrombotic microangiopathy

Microangiopathic hemolytic anemia (MAHA) is the laboratory hallmark of a host of thrombotic microangiopathies (TMAs). Included in this spectrum are TTP and HUS, but a similar blood film and potentially similar symptoms may be seen in multiple other conditions, such as malignant hypertension, preeclampsia, HELLP syndrome (hemolysis, elevated liver enzymes, low platelets), antiphospholipid syndrome, and sepsis. Although these diseases may have overlapping presenting signs and symptoms, their treatments differ, such that accurate diagnosis is essential. TTP pathogenesis is discussed in Chapter 8.

Atypical HUS (aHUS) may present with a constellation of findings similar to TTP. This disorder is usually driven by failure of control of the alternate pathway of complement activation. Supporting data include the finding of loss-of-function mutations in genes encoding for complement regulation (Factor H, Factor I, and membrane cofactor protein) or gain-of-function variants on complement factor B and C3 in as many as 50% of patients with aHUS, the presence of anti–Factor H autoantibody in a minority of patients, and the successful amelioration of aHUS through administration of anticomplement monoclonal antibodies. Finally, typical HUS develops as a consequence of infection with enteropathic *Escherichia coli* that produces a Shiga-like verotoxin often presenting with abdominal symptoms, such as abdominal pain and bloody diarrhea, but is followed several days to a week later with organ dysfunction (such as acute kidney injury) and MAHA due to systemic effects of absorbed toxin.

TTP is distinguished from the other TMA syndromes by the finding of severe deficiency of ADAMTS13, and levels are typically <10%. Measurement of ADAMTS13 level as well as assay for markers of an autoimmune mechanism are important at initial presentation of TTP, and follow-up activity assays are becoming important for the ongoing management of patients with idiopathic TTP (iTTP) due to autoantibodies.

The main laboratory methods that are currently used employ a recombinant 73–amino-acid peptide from the A2 domain of VWF (VWF73 peptide) containing the Y1605-M1606 bond recognized by ADAMTS13 to detect substrate cleavage by either ELISA, fluorescence resonance energy transfer (FRET), or chemiluminescent methods. Two amino acids in the peptide substrate are modified in the FRET assay; one fluoresces when excited, and the other absorbs or quenches the released energy. When ADAMTS13 cleaves the substrate and separates the modified amino acids, emitted energy is detected in a fluorescent plate reader. Alternatively, a chromogenic endpoint assay is available in which the VWF73 cleavage is detected by a monoclonal antibody that recognizes the exposed amino acid sequence after peptide cleavage occurs. The method for ADAMTS13 neutralizing antibody detection is similar to the Bethesda assay for factor VIII inhibitors; dilutions of patient serum or plasma are mixed with PNP, which is followed by measurement of residual enzyme activity using the synthetic substrate. Typical reference values are ADAMTS13 activity >67% and inhibitor titer <0.4. In approximately one-third of cases of iTTP, mixing studies fail to identify a neutralizing antibody. This may be either due to presence of autoantibodies that cause clearance rather than inhibition of the enzyme, the limited sensitivity of some assay methods, or low antibody titers. In such cases, ELISAs in which the ELISA plate is coated with full-length recombinant ADAMTS13 are used to detect anti-ADAMTS13 autoantibody, and reflexive testing algorithms should be considered in cases of suspected iTTP in which inhibitor assay is negative. In hereditary forms of TTP, the activity of ADAMTS13 is absent or markedly decreased, activity does not recover during clinical remission, no inhibitor is present, and cTTP may be confirmed through sequence analysis of the gene encoding for ADAMTS13 gene.

The decision about whether to initiate plasma exchange in a patient suspected of having TTP is made on the basis of clinical assessment using a validated clinical prediction tool, such as the PLASMIC score, and treatment should not be delayed while awaiting ADAMTS13 testing in the absence of a rapid test result. Importantly, samples for assessment of ADAMTS13 activity and inhibitor should be obtained prior to transfusion with fresh frozen plasma or plasma exchange; however, samples may still

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be informative after initiation of apheresis treatments if a preapheresis sample is not available.

There is increasing evidence that ADAMTS13 testing obtained following completion of a course of plasma exchange provides important information related to patient risk of early return of symptoms (termed exacerbation) or delayed relapse. Severe ADAMTS13 deficiency shortly after the completion of plasma exchange may be indication for continuation of adjunctive therapy, such as caplacizumab. Late decline of ADAMTS13 below some yet to be defined threshold has been interpreted by some practitioners as an indication for presumptive initiation of immunosuppressive therapy with rituximab to prevent relapse.

Diagnosis of typical HUS and atypical HUS and distinction for other forms of TMA are driven in part by the clinical setting. Typical HUS is more common in the pediatric population, may occur in epidemics related to contamination of the food supply, and is often but not always preceded by gastrointestinal symptoms. Diagnosis may be confirmed by either stool culture on sorbitol-MacConkey agar, immunologic identification of Shiga toxin, or nucleic acid detection of toxin structural gene message using polymerase chain reaction–based methods. Collection of sample early after clinical presentation is important, as yield decreases with time. Diagnosis of aHUS is difficult and to some extent is a diagnosis of exclusion, as early initiation of anticomplement therapy gives the best chance for renal function recovery. With the possible exception of anti–factor H serology, there are no readily available phenotypic assays to support disease diagnosis, and sequence analysis of complement genes is time consuming and unrevealing in a substantial proportion of patients.

Assays for thrombophilia

Highly sensitive D-dimer assays in combination with pretest probability scores, such as the Well's score, are frequently used in the outpatient setting (such as the emergency department) to exclude VTE. Because D-dimer is a breakdown product of fibrin clot, a negative D-dimer value (below the established cutoff) has been shown to have high negative predictive value to exclude VTE in patients with low or intermediate clinical probability. Results above the cutoff for the assay do not confirm VTE but identify patients who should undergo additional evaluation, such as imaging studies. D-dimer assays, VTE cutoffs, and reporting units vary between laboratories, and knowledge of these variables is required if D-dimer results are used for this purpose.

Inherited deficiency of 1 or more of the identified natural inhibitors of coagulation (AT, PC, and PS) is a risk factor for venous thrombosis, and functional and immunologic assays are available to measure these inhibitors. The use of these assays generally should be restricted to patients in whom the result may affect prognosis and duration of anticoagulant treatment. This generally includes patients who present with spontaneous thrombosis not temporally related to recent surgery, trauma, immobilization, cancer, or other acquired risk factors. The likelihood of identifying a deficiency is increased if thrombosis is recurrent or in an unusual location, the patient is young (<45 y old), or the patient has a positive family history of thrombosis. To avoid misleading low results due to temporary conditions related to acute illness, thrombosis, and anticoagulant therapy, testing for non–genetically based assays ideally should be delayed until several weeks after completion of treatment when a patient has returned to baseline. The biologic and analytical variability associated with phenotypic diagnoses of these deficiencies requires verification of an abnormal test result on a new sample. Because of the large number of mutations associated with deficiencies of AT, PC, and PS, genotyping is not routinely performed.

Antithrombin deficiency

Antithrombin is a serine protease inhibitor (serpin) that controls the coagulation process by inhibition of thrombin as well as coagulation factors IXa, Xa, XIa, and XIIa. AT activity is increased 1000-fold by heparin. The most sensitive screening tests for AT deficiency are chromogenic activity assays designed to quantify AT inhibition of factor Xa or IIa in the presence of UFH. Abnormal low AT activity results should be repeated and may be followed by the measurement of AT antigen to classify the deficiency as type I (activity = antigen) or type II (activity < antigen); however, the clinical significance of subclassification is unclear. Type I AT deficiency is more common than type II deficiency in symptomatic kindreds. Subclassification of type II deficiency requires performance of the chromogenic activity assay without heparin to differentiate type IIa resulting from reactive site defects and IIb resulting from AT heparin-binding defects. Although type IIb is associated with a low risk of thrombosis, progressive AT activity assays are not readily available and typically not performed. Heterozygous AT deficiency is identified in approximately 4% of families with inherited thrombophilia and approximately 1% of consecutively treated patients with an initial episode of venous thromboembolic disease. Low AT values are typically seen in patients on heparin therapy because of consumption.

Protein C deficiency

Protein C is a vitamin K–dependent zymogen, which once activated controls coagulation through cleavage of

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coagulation factors Va and VIIIa. The preferred screening test for PC deficiency is a chromogenic activity assay. PC is activated with a snake venom, and PC activity correlates with hydrolysis of a synthetic peptide and change in OD. Clot-based PC activity assays are an alternative, but potentially inaccurate results may occur due to variations in factor VIII and PS levels, FVL, inhibitory antibodies, and presence of some anticoagulants. Abnormal low PC activity results should be repeated and may be followed by measurement of the PC antigen to classify the deficiency as type I (activity = antigen) or type II (activity < antigen); however, the clinical significance of subclassification is unclear. Chromogenic assays detect type I deficiency as well as type II defects if that defect is in the catalytic active site, whereas clot-based assays have the potential to also detect abnormalities in additional protein C domains.

Protein S deficiency

PS assays are challenging because of the unique biology of PS. Total plasma PS is partitioned between free and bound forms. The protein is nonfunctional when bound to complement 4b–binding protein and functional when it is free. In its unbound form, the protein can serve as a cofactor for activated PC (aPC). The typical PS bound-to-free ratio of 60:40 varies under different physiologic and pathologic conditions. Decreases in free/functional PS are seen in acute phase conditions and during pregnancy. Clot-based PS activity assays are the most sensitive screening tests for PS deficiency but suffer from potential inaccuracy because of the same variables that can affect PC activity testing. An alternative screening assay is free PS antigen concentration to avoid confounding variables. Free PS testing, however, is insensitive to type II PS deficiency (low activity but normal free antigen level). Some laboratories screen with PS activity, some screen with free PS antigen, and other laboratories use both assays. Because of inaccuracy in diagnosis, the true prevalence of protein S deficiency remains unknown.

Factor V Leiden and prothrombin gene mutations

Two autosomal inherited coagulation factor variants increase the risk for VTE; these are factor V G1691A (FVL) and prothrombin G20210A. Several sensitive commercial clot-based screening assays for FVL mutation demonstrate a resistance of factor Va cleavage by aPC in the presence of FVL mutation. Coagulation testing activated with aPTT, PT, or Russell's viper venom reagents is performed with or without added aPC, and the clotting times are expressed as a ratio. Abnormally low ratios represent aPC resistance (aPCr). Specificity is improved by repeat testing of positive plasmas after dilution with factor V–depleted plasma to minimize impact of deficiencies, anticoagulants, and high factor VIII levels. Genotyping should be performed on all aPCr-positive patients to determine whether they are heterozygous or homozygous for FVL. Although prothrombin G20210A mutation is associated with elevated prothrombin levels, measuring factor II activity is not a sensitive screening test, and genetic testing is the primary method. Both FVL and the prothrombin gene mutation are Caucasian founder-effect mutations. The prevalence of heterozygous FVL is 3% to 8% in Caucasian populations and about 1.2% in persons of African American heritage. The prothrombin gene mutation is detected in approximately 2% of US citizens and 0.5% of persons of African American heritage.

Antiphospholipid syndrome

APS is an important acquired thrombotic condition. Consensus-based criteria have been developed for the investigational classification of APS. These criteria require a combination of clinical conditions (unexplained venous or arterial thromboembolic events, pregnancy morbidity) and persistent laboratory evidence of autoantibodies that recognize epitopes on selected proteins associated with phospholipids and identified by coagulation-based (LACs) or serologic-based (aCL and anti–β_2-glycoprotein IgM and IgG antibodies) testing. LACs are heterogeneous antibodies that interfere with in vitro clotting assays. Evidence for the presence of an LAC requires (i) prolongation of a screening clotting assay designed to be sensitive to the phospholipid-dependent behavior of LAC, (ii) ruling out prolongation due to a coagulation deficiencies by showing incomplete correction in a 1:1 mix of patient and normal pooled plasma (which would correct deficiencies), (iii) confirming phospholipid dependence by shortening the clotting time with the addition of more phospholipid, and (iv) exclusion of other coagulopathies, such as a specific factor inhibitor, which may demonstrate similar laboratory patterns. Although some LACs are discovered when a routine aPTT is prolonged, a normal aPTT is generally not a sensitive LAC screening test and should not prevent performance of more sensitive LAC testing based on the clinical circumstances. There is no gold-standard assay for determination of the presence of LAC. Recent updated consensus expert guidelines from the International Society of Thrombosis and Hemostasis Scientific Subcommittee on Lupus Anticoagulant/Phospholipid Antibodies and Clinical and Laboratory Standards Institute recommend performing 2 sensitive LAC tests in parallel—1 aPTT-based test and 1 Russell's viper venom (activation of factor Xa)-based test—and accepting a positive result from either or both as evidence of an LAC. Preanalytical

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variables requiring attention include platelet contamination (>10,000/mL) due to inadequate centrifugation, which can produce FN LAC results because of the neutralizing effect of platelet-derived phospholipid, and concurrent anticoagulation therapy. The presence of a DOAC in the test plasma nullifies the validity of LAC testing. The use of DOAC adsorbents is a potential procedure that is still considered investigational. Heparin can be neutralized by additives in the LAC test reagents or in a separate step before testing, and the mixing step can compensate for mild to moderate coagulopathies due to liver disease or vitamin K antagonists like warfarin. The preferred time, however, for LAC testing is before initiation or after completion of anticoagulation treatment. Although testing of a patient on anticoagulant therapy is discouraged, if this is clinically necessary, it is important to make the laboratory aware of the specific anticoagulant drug that the patient is receiving in order for the lab to provide appropriate interpretation of the generated coagulation data. For some patients, temporary discontinuation of anticoagulant therapy (or switch to LMWH) may be acceptable in order to allow for accurate assessment for the presence of LAC. Rarely, a specific factor inhibitor can cause an FP LAC result, typically with an aPTT-based LAC test due to a factor VIII inhibitor. A more frequent occurrence, however, is the appearance of multiple coagulation factor deficiencies when the true coagulation factor levels are within normal limits; this misleading picture occurs because the same antibodies responsible for the LAC effect also interfere with phospholipid support of coagulation factor assays. The hematologist should be aware that rare patients concurrently may have both an LAC and a true factor VIII inhibitor. Abnormal bleeding likely would be present, and specific factor assays would confirm an isolated factor deficiency. LAC tests are either positive or negative, and evidence is insufficient to support reporting gradations of positive results. Because of differences in test methods, reagents, instrumentation, preanalytical variables, and approaches to analyzing and reporting results, there is substantial interlaboratory variability of LAC results as revealed by external proficiency testing surveys.

LAC can cause reagent-dependent prolongations of PT results. Although this is usually mild, occasionally LAC-positive patients have elevated INRs before starting warfarin. Chromogenic factor X activity (not chromogenic anti-Xa) is an alternative to the INR for therapeutic anticoagulation monitoring (target 20% to 40%); however, availability of the test is limited. Another option is to measure PT-based factor II, VII, and X activities and observe whether the LAC produces an inhibitor pattern on the serial dilutions of plasma. If 1 or more factor assays appear unaffected by the LAC, then suppression of a specific clotting factor can serve as the therapeutic target for warfarin anticoagulation. A markedly prolonged PT in the setting of LAC may be a result of acquired factor II deficiency due to a nonneutralizing prothrombin autoantibody that increases the clearance rate. These patients with lupus anticoagulant hypoprothrombinemia syndrome are at risk for spontaneous bleeding. To recognize this rare condition, a factor II activity level should be obtained in an LAC-positive patient with a prolonged PT/INR.

Performance of immunoassays for aCL and anti–β_2-glycoprotein I (aβ_2GPI) IgM and IgG antibodies should accompany LAC testing to maximize sensitivity because persistently positive (arbitrarily defined as >12 wk apart) results from serologic tests or LAC or both fulfill the laboratory criteria for APS. Commercial ELISA kits and chemiluminescent assays for aCL and aβ_2GPI lack standardization, and interlaboratory agreement is poor for weakly positive sera. To improve specificity, some experts consider only medium- and high–titer-positive IgG and IgM aCL and aβ_2GPI results (≥40 IgG phospholipid units or IgM phospholipid units) to be clinically important. In addition, testing during significant immunosuppression (especially humoral) may lead to FN results. Although other antiphospholipid antibody specificities are currently not included in the classification criteria, antibodies to aβ_2GPI domain I and antiphosphatidylserine/prothrombin antibodies have been shown to be predictive of thrombotic risk.

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